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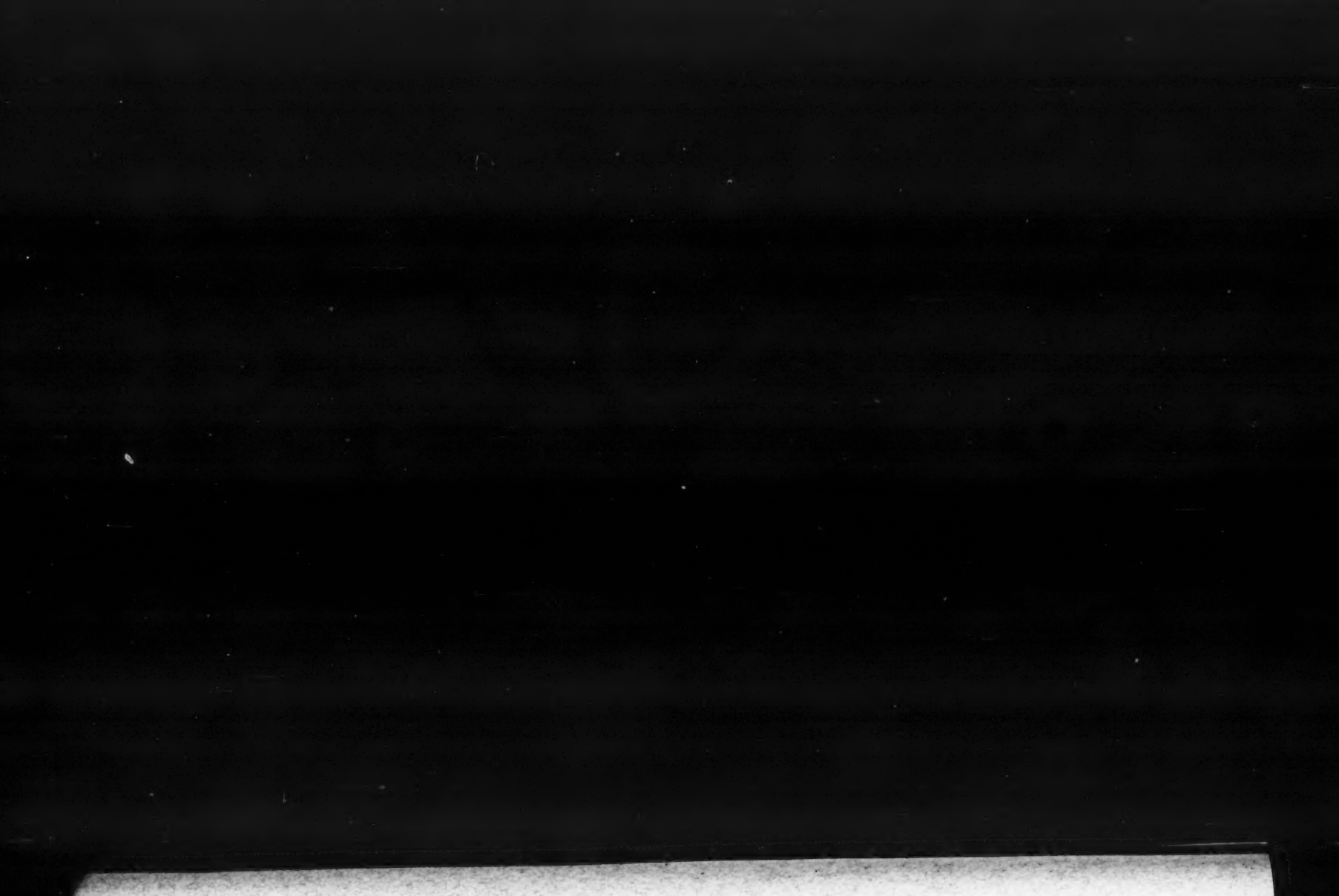

Vol. XLIX *November, 1925* *No. 5*

BIOLOGICAL BULLETIN

THE RELATION OF *HERPETOMONAS ELMASSIANI* (MIGONE) TO ITS PLANT AND INSECT HOSTS.

FRANCIS O. HOLMES.[1]

Herpetomonas elmassiani (Migone) is at present the only species of latex-inhabiting herpetomonad known in the United States. Its plant host is the common milkweed, *Asclepias syriaca* L., in the latex of which it has been found in great numbers in Maryland and New Jersey (Holmes, 1924, 1925*a*). Its suspected insect host in these locations is a red and black hemipterous insect, *Oncopeltus fasciatus* (Dall.). The same species of plant flagellate appears to inhabit other milkweeds in countries along the Atlantic coast of Central and South America. It has been reported from Haiti, Honduras and Paraguay, and doubtless exists at intermediate points between these countries and the locations in the United States. In southern locations other species of *Oncopeltus* frequenting the infected plants have seemed to act as insect hosts.

In view of the lack of apparent pathogenicity of the herpetomonads in Maryland milkweeds (Holmes, 1925*b*), it seems desirable to report upon the relation of the flagellate to the tissues of its hosts. The well known species, *Herpetomonas davidi* (Lafont), which inhabits the latex cells of Euphorbias in Europe and elsewhere, is pathogenic to its host (França, 1914, Nieschulz, 1922), in which it causes modifications of the latex cells and neighboring portions of the plant sufficient to stunt or kill whole branches or even whole plants. The reasons for the lack of harm resulting in the milkweed host from the presence of *Herpetomonas elmassiani* (Migone) may be the freedom from infection of some of the latex systems even of heavily infected

[1] Joint contribution from the laboratories of the Boyce Thompson Institute for Plant Research and of the Department of Medical Zoölogy, School of Hygiene and Public Health, Johns Hopkins University.

plants, the entire freedom from penetration of tissues aside from the latex systems, and the sufficient food supply presented to the flagellates by the milky vacuole fluid itself in the latex cells.

The fact is often overlooked that plant flagellates of the type of *Herpetomonas davidi* (Lafont) and *Herpetomonas elmassiani* (Migone) have been found *only* in such plants as have abundant latex. Those engaged in research in this field are of course acquainted with this restriction of the range of the organisms, and take it into account in most of their work, but do not always seem to have it very definitely in mind. Others whose interest in plant flagellates arises from some other, less immediate, source are often entirely unmindful of the situation until it is brought to their attention.

To the present time no acceptable species of flagellates of the genera *Herpetomonas*, *Leishmania*, *Crithidia* or *Trypanosoma* have been found in plants other than those provided with a milky juice or latex.

Perhaps the situation would be better understood if it were commonly known that latex does not occur extracellularly in plants, but intracellularly. Thus the flagellates which are transferred by their insect hosts to the latex cells of plants are not to be found thereafter at random in the plant tissues, but are strictly intracellular (not intracytoplasmic) parasites.

A description of the cells containing the latex will make clear the relation of any latex-inhabiting organisms to the host plants.

The latex ducts of the plants with which protozoölogical studies have been most concerned are those known as simple ducts, because they do not fuse with each other in the course of their wanderings. Among the Asclepiadaceæ, Euphorbiaceæ, Apocynaceæ and Urticaceæ the cells destined to become the latex ducts of the mature plants are already distinguishable in the embryo. Their nuclei divide again and again, and the cells elongate tremendously and branch repeatedly, but no cross walls are formed, nor do the ducts fuse with one another, so that eventually the few original cells penetrate every part of the plant, and still remain distinct and separate from each other. The thin cellulose walls are lined with a layer of cytoplasm containing numerous nuclei. In the vacuole is collected the milky, usually white latex.

It is well to bear in mind that there is another type of latex system among plants, in which the original cells fuse together by the destruction of their partitions and cross-walls. The result

FIG. 1. Diagrammatic representation of a portion of a simple latex duct such as is found in the species of the genus *Asclepias*. The latex is secreted by the wall of cytoplasm into the extensive central vacuole. Such a duct will not fuse with others in the course of its growth.

of the process is the formation of interlacing vessels, in which the latex is contained. This type of latex system is found in such plants as lettuce.

Diagrams representing these two types of latex ducts are shown in Figs. 1 and 2.

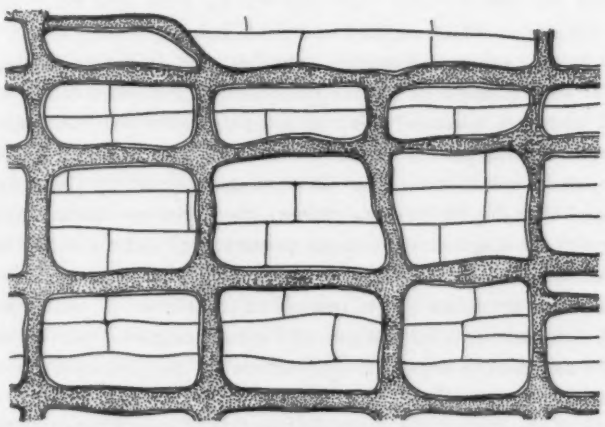

FIG. 2. Diagrammatic representation of a system of latex vessels such as is found in lettuce. The original cells fuse together to form a network, instead of remaining as independent latex ducts. The type of latex cell involved in protozoölogical studies has been that shown in Fig. 1.

The flagellates known to inhabit the latex systems of plants are probably all insect parasites. They enter the cells of the

plant hosts only when the insects introduce their beaks directly into the latex to feed. In the cells they do not enter the cytoplasm, so far as can be seen, but inhabit the milky juice which fills the long cell vacuole. There they grow and multiply rapidly, as is indicated by the many dividing specimens present in latex smears from infected plants stained with Wright's stain. The flagellates are shut off from other cells of the plant and even from other latex cells of which there may be several. Unless a single plant were infected several times by insects it is unlikely that all the latex ducts would become infected, even though one or more might.

Localized Infections.

If one latex system of a plant is infected, a macroscopically localized infection may result unless that system happens to penetrate every leaf and flowerlet of the plant.

Early in the season of 1924 a search was made for such cases, for the previous year all the infections had appeared systemic.

It seemed likely that insects might repeatedly bite plants during the late summer, but that during June and early July any infections which might occur from insect carriage would be the result of a minimum number of infective bites.

The first example of the way in which this worked out in the field was met when a plant of a group surveyed thoroughly every few days gave a negative record after showing flagellates on several occasions. The later examinations of this plant showed that flagellates were present, but were not always to be found in the single drops of latex preserved as records. For this plant, then, a new system of sampling was instituted. Samples were taken from ten leaves instead of from one. It was found that some leaves were positive and others negative, just as one might reasonably expect if only a few of the latex cells were parasitized.

The study of sections of petiole and leaf tissue gathered at this time showed the even more interesting fact that in plants never suspected of having localized infections only a few of the latex cells were inhabited by organisms. It was easier to find negative cells than positive in specimens from apparently heavily parasitized plants. Smears from these plants showed very large

numbers of organisms, yet when a droplet was taken for examination as much latex must have been contributed by uninfected cells as by infected. In the cells containing the organisms the concentration was so great that a moderate dilution was entirely ineffective in changing the appearance of the drop as stained for examination.

Since the plants above mentioned seemed when in the field to have systemic infections in that the slightest wound in any portion of the leaves or stems gave infected latex, and yet the microscopic examination of sections showed but a few of the cells infected, it is evident that the whole plants were penetrated by a relatively few single cells, and that if each individual latex cell fell short of extending completely throughout its plant, it still must have achieved very nearly this remarkable feat. This type of cell attains a notable total size and contains tremendous numbers of nuclei. Its latex-containing vacuole also is probably nearly if not quite the largest of cell vacuoles, making up in length far more than it lacks in breadth.

Confinement to Latex Cells.

Careful study failed to show any flagellates in the plant tissues outside the vacuoles of the latex cells. In some sections there were indeed abnormal and deceptive appearances caused by the flow of latex by capillary action along the conducting tissues. When pieces were cut to be fixed, latex always flowed out over the wounded surfaces. Since the turgidity of the plant was in part relieved by this loss, other specialized cells such as the spiral vessels were invaded by currents of latex along with which the flagellates themselves were carried. The abnormality of this process was made evident by examining the invaded ducts throughout their entire length. The flagellates were seen to be present only near the cut surfaces and to become less and less crowded as the distance from the exterior increased. The tissues in the interior presented a truer picture of conditions in the living plant.

Since the flagellates were normally confined to the latex cells, and indeed to the *latex* filling the long vacuoles, it became evident that certain observations made in the field were of more signifi-

cance in their relation to the biology of the flagellate than had at first been realized.

It had been noticed that infections did not spread from plant to plant in groups connected by a common axis. Such plants arose from separate buds, the latex systems of which were independent of each other. In the axis itself no ducts occur, so that there is no chance for wandering through such a connection.

Late in the autumn of 1924 two plants which had been under observation for months were dug up to determine their exact relation to each other. One of these had been consistently negative for flagellates all summer. The other had been as consistently heavily infected in every part above the ground, with the exception of the seeds which are always free from invasion. The two plants were separated from each other by no more than six inches of axis, from which common source they had both sprung as buds. The absence of latex ducts in the axis and the confinement of the flagellates to the latex of the infected plant made it impossible that the nearby negative plant should be invaded except from some outside source of the organisms.

The practical confinement of the insect, *Oncopeltus fasciatus* (Dall.), suspected of being the insect host of the flagellate, to the blossoms and pods of the milkweed plant in feeding is also made significant by histological studies of the latex system, which in these two parts becomes much more prominent than it is in the stems or leaves. The soft tissues under the outer green coverings of the pods contain numerous branches of the latex system, and in the area between the pedicels and the bases of the petals of the flowers the latex ducts are exceedingly close together and voluminous. This offers a favorable feeding location to the insect, and, by reason of the crowded flagellates here in infected plants and the softness of the tissues, opportunity is offered for the infection of insects from the plant and for the infection of previously uninfected plants by the insects.

The Flagellates of *Oncopeltus fasciatus* (Dall.).

It is not yet known with certainty whether *Oncopeltus fasciatus* (Dall.) is the insect which carries the milkweed flagellate from

one plant to another. But since there are several reasons for suspecting it to be the carrier, a study of its flagellates was undertaken. All along the coast of America, from Paraguay in the south to New Jersey in the north, wherever the flagellate has been found in the latex of the milkweeds some species of the genus *Oncopeltus* has been found feeding on the infected plants. These insects have always been more characteristic of the particular plants harboring the flagellates than any other type of insect. Just north of New Jersey, where no flagellates are known in the plants, another insect genus *replaces Oncopeltus*. The specimens of this which have been examined thus far have been entirely negative for any flagellate, though infected with Sporozoa both intracellularly and extracellularly in the salivary glands. This coincidence of the range of the milkweed flagellate with the range of *Oncopeltus fasciatus* (Dall.) suggests that the presence of the flagellate is dependent on the presence of the insect. Moreover *Oncopeltus* feeds characteristically on the pods and flowers of the plant, and since plants bearing seed are the ones to which the infection is practically limited it seems even more probable that *Oncopeltus* is the host. Another bit of circumstantial evidence is gained from the study of the morphology of the flagellates from the insect and from the plant. The plant flagellates are characterized by a twist in their ribbon-like bodies. This is rare in insect flagellates, but is found in the case of the parasites of *Oncopeltus*. The insect feeds on latex. The infected plants have swarms of flagellates in *their* latex in every portion of the upper parts of the plants. Thus the insect could easily become infected from his feeding. In the insect the histological studies about to be described have shown swarms of flagellates in definite lobes of the thoracic salivary gland. The secretion of this gland is led by a simple duct directly to the mouth parts during the process of feeding, so that there should be no great mechanical difficulty in the transfer of the insect's flagellates to the interior of the latex ducts of the plant.

It is natural then that in spite of the difficulties which have been experienced up to this time in attempting to obtain plant infections from the insects in regions where plant infections are not known in the field, efforts to establish the relation of the

plant and insect flagellates by histological studies have been made. The results have been negative so far as obtaining definite proof of the identity of the two forms is concerned, but such interesting observations have been made during the study that they will be described here.

The first insects to be sectioned and stained were collected during 1923. At first the flagellates were overlooked, and that for two very good reasons. The principal search was made for them in the intestinal tract, where they do not occur in my material, if indeed they ever occur there. And the salivary gland forms, stained as carefully as they may be, never stand out with the clearness by which those in latex smears are characterized. For the process of drying and staining with Wright's stain, though open to the objection that the nuclear detail is lost, gives bright sharp pictures of the organisms, usually making them much darker than the background. This is far from the case with wet-fixed material, sectioned, and stained even with so good a stain as iron hæmatoxylin. By this process the nucleus may be stained with all the desired sharpness, but the background of salivary secretion retains the stain far more than does the flagellate's cytoplasm. So that usually one sees the body of the flagellate only as an unstained area surrounding the nucleus. The wall of the salivary gland also retains the stain so tenaciously that it must usually be left very black that the internal structure of the flagellate may be seen at its best. This makes the whole field very dark, with the object to be examined exceedingly delicate and lightly stained. When these first specimens of *Oncopeltus* were examined a second time for a different purpose, clusters of the flagellates were noted by chance on the wall of the gland. The slide under observation at the time was stained with a mixture of aniline dyes, but the finding was at once confirmed by iron hæmatoxylin slides of the same material.

During 1924 a quantity of material was obtained for sectioning to discover the extent of the infection among the insects during the late season when the plants were becoming more and more widely infected, to determine with certainty that the organ in question was really the salivary gland of the insect and not a salivary receptacle or some other organ, to see whether the

organism present had more than one stage in its life-history in the insect, and to find exactly its relation to the salivary gland tissues and secretion.

The study of the morphology of the gland in which the flagellates occurred required complete series of serial sections of three insects. The gland of one of these was reconstructed section by section, for the lobes of the gland cannot be readily visualized from the separate sections. Sometimes but one or two show, at other times there appear to be five or six lobes because of the inclination of the section and the twists in the organ itself.

Histology of the Salivary Gland.

The salivary gland nearly fills the dorsal half of the thorax. Its efferent duct leads away from the point at which the three lobes come together, first running back toward the abdomen immediately under the gland, then turning and running forward. It dips beneath the œsophagus near its junction with the proventriculus, and beneath the ventral chain of nerve ganglia between the sub-œsophageal ganglion and the ganglion immediately posterior to it. From this point the duct runs forward to the mouthparts where its secretion enters the pump or syringe and the hypopharynx.

It was interesting to note that the three parts of the gland could be distinguished readily in iron hæmatoxylin eosin sections by the character of the secretion in each, in spite of the fact that the outline of each might be most deceptively placed according to the plane of the section. The anterior lobe contained frothy material staining pink with eosin. The ventral lobe was distended with a smooth or very slightly granular substance which also stained pink with eosin. It was thus easy to distinguish these two by the consistencies of their contents. The dorsal lobe was remarkable in that its fluid retained some of the iron hæmatoxylin stain and in addition picked up eosin eagerly, nearly attaining the brilliant orange red color characteristic of blood corpuscles in tissues stained for example with Delafield's (not Heidenhain's) hæmatoxylin and eosin.

Localization in Dorsal and Anterior Lobes.

It was in the third or dorsal lobe that the flagellates were really abundant. There they were often massed like a new tissue lining the entire lumen of the gland. The dark stain which the fluid took made it difficult to study the internal structure of the organisms. Yet they were so abundant that some were usually favorably located for observation. The flagellates lined the dorsal lobe most heavily near its posterior extremity. Nearer the efferent duct the organisms were often in groups or clusters, isolated a little from the rest of the mass, but closely bunched together with flagella attached to the wall.

In the anterior, foam-filled lobe there were a few flagellates always when they were present in the dorsal lobe. In the posterior lobe there were none, no matter how heavily the insect was parasitized.

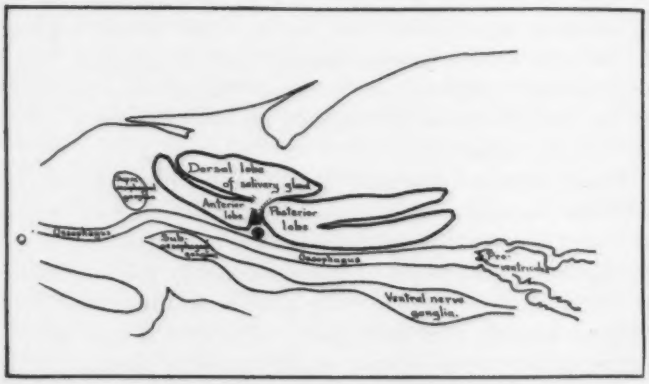

FIG. 3. Portion of a longitudinal section through *Oncopeltus*, showing the relative positions of the salivary gland and other thoracic organs. The three lobes of the gland come together in this particular section and the point of entrance of the salivary duct is shown.

That the fluids from the three lobes intermingled slightly at the common point of contact where the three fluids drained into the same efferent duct was indicated by the mixed consistencies and colors just at this exit point. In the smooth orange-red fluid a cloud of pink was always seen to have penetrated for a

short distance. In the two pink portions, granular and frothy, red clouds were also visible just at the same point.

Until the gland had been reconstructed by drawing each section on blotting paper, cutting out and pasting together the individual sections, and coating the whole model with a beeswax and paraffin mixture, it was impossible to get an adequate idea of the complete gland. The lobes were so complicated in some planes of the section that it was not certain that no salivary reservoir entered the question. As the work was completed, however, it became evident that the structure in which the flagellates were located was a three-lobed thoracic salivary gland, with a single common exit. A diagram of the longitudinal section through the opening of the three lobes is to be seen in Fig. 3.

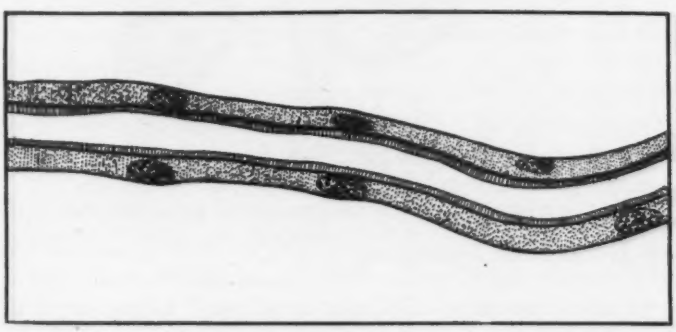

FIG. 4. A portion of the efferent duct of the salivary gland of *Oncopeltus*.

The cells of the duct are characteristic and interesting and with the secreting cells of the gland are so readily recognizable that no difficulty arises in identifying them even in sections which show but a tiny fragment of the salivary apparatus.

The duct is lined with a single layer of cells the nuclei of which are sometimes branched. A portion of the duct is represented in Fig. 4. A similar detailed drawing of the cells of the wall of the salivary gland is shown in Fig. 5. The glands are commonly distended with fluid. This escapes gradually, as required, through the efferent duct.

With the recognition of this organ as a thoracic salivary gland

one of the objects of the histological examination of the insects was accomplished.

It was interesting to note that the flagellates always developed in the dorsal lobe most abundantly. A few penetrated the anterior, but none the ventral lobe. Evidently the differences in the compositions of the salivary secretions of the three lobes, indicated at once by the different staining reactions of the three portions, had also a significant effect on the organisms, inducing them to inhabit one portion more than others, and excluding them from the ventral lobe.

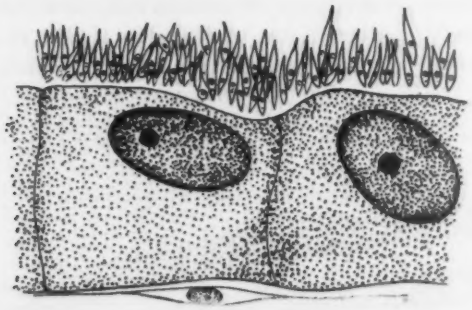

FIG. 5. A portion of the wall of the dorsal lobe of the salivary gland of an infected *Oncopeltus*. The nuclei of the gland cells are gigantic in comparison with the minute nuclei of the flagellates.

In my material no intestinal forms were found. This is remarkable when one considers that the type species of the genus *Herpetomonas* is a strictly intestinal flagellate in the common housefly. Salivary gland forms are the exception rather than the rule among Herpetomonads, and it would be expected that intestinal forms of the flagellate would be more noticeable than the gland forms. But if the flagellates of *Oncopeltus fasciatus* (Dall.) ever show intestinal forms I have not been able to secure the proper material to demonstrate them.

In addition to the complete series of sections of three individuals of *Oncopeltus fasciatus* (Dall.), a group of ten of the same species was sectioned and studied by choosing representative sections for staining and for examination. Sixteen individuals of *Lygæus kalmii* from Massachusetts were treated in the same way.

The object of using *Lygæus* was this: the limit of the range of *Oncopeltus fasciatus* coincides with the limit of the range of the plant flagellate, *Herpetomonas elmassiani* (Migone). But *Lygæus* is a very closely related insect, replacing *Oncopeltus* in the north where the flagellates are not found. It was desired to know whether it also had flagellates in its salivary glands. The thirteen specimens of *Oncopeltus*, which came from Maryland in September, 1924, at a time when the spread of plant flagellates was going on rapidly, were all infected with the exception of three individuals, which were rather young nymphs. The sixteen specimens of *Lygæus* were all negative for flagellates, both in the intestinal tract and in the salivary gland.

In the *Lygæus* examined there *were* infections with sporozoa in the glands, curiously enough in the same lobes frequented by the flagellates of *Oncopeltus*. One of the species present in *Lygæus* was occasionally seen also in *Oncopeltus* along with the flagellates. But in *Lygæus* the infections were much heavier, and decidedly destructive to the gland cells which were penetrated by intracellular stages and often rendered useless for secretion by the growth of the parasites and the consequent death of the cells.

The absence of flagellates in sixteen specimens of *Lygæus* collected at the end of the season was strikingly in contrast with the presence of large numbers of flagellates in the glands of ten of the thirteen *Oncopeltus* sectioned. If *Oncopeltus* is responsible for the spread of the milkweed flagellate it is no wonder that the spread is very rapid in September and early October when so large a percentage of the then rapidly multiplying insects are positive.

Of the original problems for the solution of which the histological work on *Oncopeltus* was carried out, but one remains for consideration. Does the flagellate of *Oncopeltus* have more than one stage of its life history in the insect? It seemed likely at first that there would be developmental stages of the herpetomonad in the intestine. But no colonization of the intestinal tract was indicated by any of my material. All the organisms were in the glands. The question then arose as to the exact position of the parabasal body in the forms colonizing the walls of the salivary gland. Careful study showed that in all cases

in which the relative positions of nucleus and parabasal body could be definitely determined, the organism was constantly a herpetomonad. Smears of the insect flagellate confirmed this, for in them the position of the organelles could be determined in every single case far more readily than in sections of the gland. This question is not quite safely settled, for at some other season of the year the evidence might differ from that which I have been able to gather, but with the knowledge of the exact part of the insect parasitized, the dorsal and anterior thirds of the thoracic salivary gland, and the extent of infection during the season at which the insects are busily feeding on the infected plants, it will be easier to work on the questions concerned with the insect host of the flagellate.

SUMMARY.

Histological studies of the milkweed host of the flagellate *Herpetomonas elmassiani* (Migone) showed that the organisms were confined to the latex system, in which they were intracellular but not intracytoplasmic. The latex is secreted into the general cell vacuole of the latex duct, and it is in this that the organisms were found. No other cells or parts of cells were found to be penetrated.

During the early part of the summer one or a very few latex cells in a plant were sometimes infected, for in *Asclepias* the original latex cells of the embryo never fuse. Because of this condition occasional localized infections appeared, in which a few leaves of the infected plant were found to be free from organisms.

The flagellates of *Oncopeltus fasciatus* (Dall.), a red and black hemipterous insect suspected of being the insect host of *H. elmassiani* (Migone), were found to inhabit the three-lobed thoracic salivary gland. In the gland these were definitely localized, colonizing only the dorsal and anterior lobes.

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HEAT PRODUCTION BY THE EGGS OF *ARBACIA PUNCTULATA* DURING FERTILIZATION AND EARLY CLEAVAGE.

CHARLES G. ROGERS AND KENNETH S. COLE.[1]

The general problem of development has in it so much of the unexplained that any attempt to add to our information by attacks from new directions may seem warranted. The work here reported was first undertaken several years ago, but was not carried to a successful issue until this past summer because of experimental difficulties unforeseen at the start.

The work was originally undertaken in an attempt to check the work of Warburg and of Loeb and Wastenys concerning the oxygen consumption of eggs before and after fertilization. It will be recalled that these investigators had found that immediately after fertilization there occurs a remarkable increase in the rate of oxygen consumption, amounting to 4 to 6 times the amount used before fertilization. It is to be assumed that there is also a corresponding increase in the carbon dioxide production of the eggs. This latter would, of course, be exceedingly difficult to measure in the case of marine eggs. If there is any considerable increase in oxygen consumption following fertilization there should be a corresponding increase in the amount of heat produced by the eggs as a result of the oxidation process. The question to be faced was whether, with the facilities at our command, we would be able to make a series of measurements which would bear examination by a physicist. Preliminary tests made in 1920 and 1921 indicated that the production of heat by fertilized eggs was a measurable quantity. These tests, observed in the latter year by a group of physicists who were visiting the Marine Laboratory, proved to be sufficiently encouraging to warrant further expenditures of time and money.

[1] Contribution from the Zoölogical Laboratory of Oberlin College, and from the Marine Biological Laboratory, Woods Hole, Mass. To the Director and other officials of the Marine Biological Laboratory we express our thanks for many courtesies extended to us.

Since this investigation was first projected others have made studies of a somewhat similar character. Myerhof (6) measured the heat production of segmenting Echinoderm eggs by means of a Beckmann thermometer, using a large vacuum flask for his calorimeter. Certain irregularities that occur in his curves may be due to experimental error, but suggest changes in the rate of heat production at different stages of development. Since then Shearer (7) has carried out similar measurements in connection with his work on the oxidation processes of Echinoderm eggs and finds that the rate of heat production after fertilization is constant for at least ten or twelve hours. He makes the statement that readings were taken at fairly frequent intervals at the commencement of the experiment, and at intervals of several hours after that. In view of this statement it seemed advisable to us to repeat the work making frequent readings and using methods of higher precision.

Possible Sources of Error.

In an investigation involving the measurement of such slight temperature changes as are expected here care must be taken to foresee and provide against all possible heat transfers into or out from the experimental flasks or to know the magnitude of such transfers. In any event such heat losses or gains should be small as compared with the total heat production which it is desired to measure. In our experiments the following possible channels of heat transfer existed, and were checked: Conduction to or from the water in the flasks

(1) by the air in the mouth of the flask,
(2) by means of the glass forming the neck of the flask,
(3) along the main thermopile,
(4) along the secondary thermopile,
(5) along the tubes of the stirring apparatus,
(6) along the fertilizing tube,
(7) by changes in the temperature of the water surrounding the flasks.

There are also possibilities of error arising from lack of care in controlling the conditions environing the electrical apparatus. Among these may be mentioned:

1. Mechanical jarring of the galvanometer used in making the measurements.
2. The temperature of the room must be maintained as nearly constant as possible during the course of an experiment. It was found to be especially necessary to avoid the possibility of drafts of air striking the apparatus.
3. It was found necessary to shield the electrical apparatus from stray electrical currents. This was found to be of the greatest importance following a severe electrical storm.
4. It was found wise to avoid all stress and strain in the wires of the thermopiles, such as might be caused by too much bending of the wires, or placing tension upon them.

It was found possible to obviate much of the possibility of the errors of the first group by using a water cap, designed so as to provide a current of water coursing continuously over the experimental flask as well as around it.

Apparatus and Methods of Work.

The general method employed was that of the micro-calorimeter, developed by Hill in his work upon muscle. Fig. 1 shows the experimental set-up used. This method has one serious disadvantage. Since corrections for heat loss are dependent upon the temperature difference and the time, long runs can not be made, since these corrections soon become a very large part of the result. In this work the corrections could be kept less than 15 per cent. of the total temperature change during a period of two and a half or three hours. Two straight sided commercial vacuum flasks of about 75 cc. capacity were used, (I.) containing 50 cc. of the egg suspension, and (II.) an equal quantity of water. These flasks had been especially exhausted through the kindness of Dr. W. R. Whitney of the Research Laboratory of the General Electric Company. When flask (I.) contained 50 cc. of water it had a heat loss of 16 calories per hour per degree difference in temperature between the interior and the exterior. The flasks used by Hill (2) had a loss of about 12 calories per hour for 250 cc. and those used by Shearer (7) about 19 calories per hour for 800 cc. This small loss for a flask of such small capacity is evidence of the great value

of very careful evacuation. The flasks were submerged in running sea water to within a short distance of their tops, and a water cap (*d*) made according to a design by White (9) was

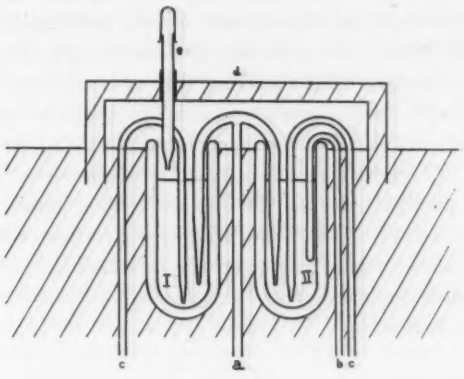

FIG. 1. Diagram of experimental set-up. I. and II., Vacuum flasks. *a*, main thermopile. *b*, auxiliary thermopile. *c*, *c*, stirring tubes. *d*, water cap. *e*, sperm pipette.

placed over them. A pipette (*e*) projected through the cap and was arranged so that "dry sperm" could be held in it, and then mixed with the contents of flask (I.) whenever desired. A continuous flow of water was maintained through this cap so that the whole formed a uniform temperature enclosure. The sea water temperature remained fairly constant except for periods of about four hours after sunrise and sunset. The variation of temperature never exceeded 0.001° C. during the period occupied by an experiment.

STIRRING APPARATUS.

It has been noted by many investigators that eggs of Echinoderms must not be heaped upon each other if normal development is to take place. In our work it was desirable to use as large a number of eggs as possible in order to get the largest possible temperature change. It was therefore necessary to devise some method of stirring the eggs which would render it impossible for them to settle to the bottom of the flask and remain there for

any considerable length of time. By the more constant stirring the eggs would at all times be able to get their needed supply of oxygen and to get rid of carbon dioxide. Work previously done by one of us (R.) had shown that stirring the water in the experimental flasks once every two or three minutes by means of an ordinary pipette was sufficient to allow the eggs to go through a normal cleavage. Such stirring was not sufficient to ensure that the water in the flask would be of uniform temperature throughout. It seemed necessary, therefore, to devise some automatic stirring device which would keep the water thoroughly stirred at all times during the course of an experiment. As a matter of interest it was found during the course of the experimentation that a failure of the stirring apparatus for as much as three minutes could be detected by a marked variation in the galvanometer readings.

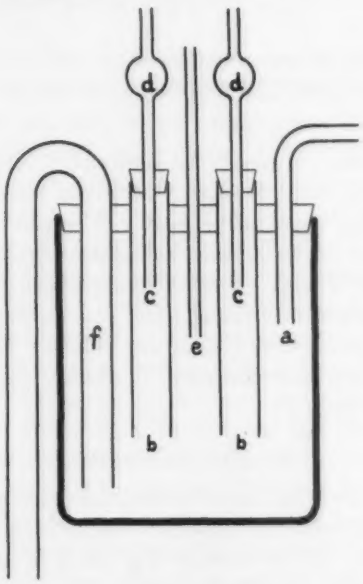

FIG. 2. Stirring apparatus. Details described in text.

The stirring was accomplished as follows: Saturated air was bubbled from the jets (*c*, *c*) at the bottoms of the flasks, by

means of the automatic intermittent siphon device shown in Fig. 2. A steady stream of water flowed in at (*a*) and as the water level rose in the bottle, air was trapped in (*b*, *b*) and forced out through (*d*, *d*). When the water reached the lower end of (*e*) it rose in the tubes, but not in the bottle (since the stopper was air tight) until (*f*) was filled beyond the bend. Then (*f*) acted as a siphon and emptied the bottle, allowing (*b*, *b*) to drain, and finally draining itself. Then the process started over again. Short pieces of rubber tubing cut at an angle were slipped over both ends of (*f*) and the lower ends of (*b*, *b*) to help in breaking the meniscus and draining these tubes. The quantity of air sent over each time could be varied by adjusting the height of the small tubes (*c*, *c*) in (*b*, *b*), and the period of the apparatus could be changed by raising or lowering (*e*). The small bulbs (*d*, *d*) prevented drops of water from being forced over into the wash bottles. These wash bottles had inlet tubes of small cross section, and were submerged in the same bath with the flasks. They served to complete the saturation of the air, if it were not already saturated, to bring it to the temperature of the bath before it went into the flasks through (*c*, *c*), and to act as a trap, preventing the suction of liquid out of the flasks through (*c*, *c*) when the stirrer bottle was emptying. In this work 7 cc. of air was sent through each flask every fourteen seconds. During a six-day continuous run, this apparatus did not vary from its average period by more than 2/5 second, always delivering the same and equal quantities of air to the flasks.

Electrical Equipment.

The electrical equipment used in these experiments consisted of the main and secondary thermopiles, galvanometer, potentiometer, storage battery cells, Weston Standard Cell, switches, etc. The main thermopile (*a*, Fig. 1) was composed of twenty pairs of copper-constantan junctions, and gave 733 microvolts per degree Centigrade, by direct calibration. (The constantan wire used was that manufactured by the Driver-Harris Wire Company, and sold under the trade name "Advance.") The reason for the unexpectedly low value of the thermopile was not found. The junctions were enclosed in thin glass tubes

filled with naphthalene and the lag of the couple was less than twelve seconds. The auxiliary or secondary thermopile (*b*) had five pairs of junctions and gave the temperature difference between flask II. and the surrounding bath. The e.m.f. developed by the couples was measured by a "White" potentiometer. This instrument, manufactured by Leeds and Northrup, gives dial readings by single microvolts and is so arranged that the resistance in the galvanometer circuit remains constant. This fact makes it possible to read fractions of a microvolt with the galvanometer. In this work one microvolt gave a deflection of 18.5 mm. on the galvanometer scale, so there were about 13,550 mm. per degree. With the high magnification telescope used it was easily possible to estimate fifths of a millimeter on the galvanometer scale. The galvanometer was of the D'Arsonval type, also manufactured by Leeds and Northrup. Its resistance was 13 ohms, period 5 seconds, and sensitivity 10^{-8} amperes per cm. with the scale a little over three meters distant. The total resistance of the galvanometer circuit was 53 ohms, which was an ohm more than the critical damping resistance. In spite of this fact there were never any oscillations, even when the sperm were introduced into the experimental flask,—only what seemed to be a very steady and somewhat rapid rise. The galvanometer circuit was shielded as far as possible, as suggested by White (10) to prevent the entrance of leakage currents into the circuit. It was, however, impossible to shield the thermopiles effectively since their leads were carried through the running sea water and very strange and erratic e.m.f.s were introduced into the circuit if the shielding system for the potentiometer and galvanometer had anything to do with the salt water or any of the piping in the room. Care had to be taken not to use for connections any wire that had been unduly bent or in any other way maltreated so as to destroy its homogeneity. On the whole the electrical apparatus worked very satisfactorily.

It was our original plan to use Hill's ingenious application of vacuum flasks to the twin calorimetric method (2) but it was found that for our small flasks the heat loss did not remain even approximately constant as the volume of the contents was varied. It was also impossible to obtain accurate values for the

conduction coefficients from cooling curves for large intervals, since Newton's law of cooling applies to small and constant temperature differences, *i.e.*, equilibrium conditions. This requirement could not be met since there was not available any current measuring device of sufficient accuracy to allow the use of electrical heating. It was necessary therefore, to get the cooling corrections under the actual experimental conditions. Following White (9)

Let θ_1 = temperature of flask (1),
θ_2 = temperature of flask (2),
θ_3 = temperature of external bath.

The temperature coefficient of conduction for the flask is defined as the temperature change between the inside and the outside of the flask in unit time when the temperature difference between the inside and the outside is unity. This may be written in mathematical form for flask (I.), $K_1 =$; and for flask (II.), $K_2 =$.

$$K_1 = \frac{1}{\theta_1 - \theta_3} \cdot \frac{d\theta_1}{dt},$$

$$K_2 = \frac{1}{\theta_2 - \theta_3} \cdot \frac{d\theta_1}{dt}.$$

Similarly, for the heat conduction between the flasks along the thermopile,

$$k_1 = \frac{1}{\theta_1 - \theta_2} \cdot \frac{d\theta_2}{dt},$$

$$k_2 = \frac{1}{\theta_2 - \theta_1} \cdot \frac{d\theta_2}{dt}.$$

Also let w_1 and w_2 equal the temperature changes due to stirring and evaporation. Then considering temperature changes where there is no liberation of heat in either flask

$$\frac{d(\theta_1 - \theta_2)}{dt} = K_1(\theta_1 - \theta_3) - K_2(\theta_2 - \theta_3) + k_1(\theta_1 - \theta_2) - k_2(\theta_2 - \theta_1) + w_1 - w_2 \quad (1)$$

$$= (K_1 + k_1 + k_2)(\theta_1 - \theta_2) + (K_1 - K_2)(\theta_2 - \theta_3) + w_1 - w_2.$$

$\theta_1 - \theta_2$ is proportional to θ_a, the e.m.f. of the main thermopile,
$\theta_2 - \theta_3$ is proportional to θ_b, the e.m.f. of the auxiliary thermopile.

So we may write

$$\frac{d\theta_a}{dt} = K_a\theta_a + K_b\theta_b, \tag{2}$$

since both theoretically and experimentally, $w_1 - w_2 = 0$ in (1). If the quantity of heat H is liberated in (1) and the heat capacity (water plus the water equivalent of the flask) is c, then

$$\frac{d\theta_a}{dt} = \frac{1}{c}\frac{dH}{dt} - K_a\theta_a - K_b\theta_b$$

and

$$\begin{aligned} H &= c\left\{\int_{\theta_0}^{\theta_a} d\theta + K_a\int_0^t \theta_a dt + K_b\int_0^t \theta_b dt\right\} \\ &= c(\theta_a - \theta_b) + c\left\{K_a\int_0^t \theta_a dt + K_b\int_0^t \theta_b dt\right\}, \end{aligned} \tag{3}$$

where θ_0 is the value of θ_a when $t = 0$.

Independent runs were made to determine the values of K_a and K_b. With θ_b small as compared to θ_a, $d\theta_a/dt$ was determined over the range for the values of θ_a used and found to be linear in θ_a. The same was done for θ_b with θ_a small. With 50 cc. in both flasks the values obtained were

K_a = .0046 microvolt per minute per microvolt difference,
K_b = .0014 microvolt per minute per microvolt difference.

The water equivalent of the flasks was found to be 8 cc. so that c = 58 cc.

A Typical Experiment.

The preparations for an experimental determination of the heat production of the eggs of *Arbacia* involved a variety of considerations not usual in ordinary experimental work in zoölogy. By careful tests the running sea water of the laboratory had been found to be the most satisfactory form of available thermostat, the temperature of the water changing only very slightly during any experimental period—usually only in thousandths of a degree. Care was, therefore, taken to have all glassware and implements used at the temperature of the sea water. Flasks, pipettes, graduates, beakers, finger-bowls, dissecting instruments, wash bottles of the stirring apparatus, thermopiles, water-cap and the animals to be used were all left in running sea water of uniform temperature for some time before

the beginning of the experiment. Whenever possible all of the eggs used in an experiment were taken from a single female. These were allowed to stand for a few minutes in a finger bowl in a little more than 50 cc. of sea water. The finger bowl was floated in running sea water. Remnants of the ovary and other debris were removed from the finger bowl by forceps or pipette. After a few minutes the eggs were stirred in the water so as to be evenly distributed throughout the whole mass of water and exactly 50 cc. of the suspension was transferred to flask (I.) by means of a volumetric pipette. Also 50 cc. of sea water were placed in flask (II.). The temperature of flask (II.) was then made enough higher than that of flask (I.) so that at the end of the run it would be about as much below (I.) as it was above at the beginning. This made the value of the integral involving θ_a approximately zero and kept the maximum value of the corrections low. The water-cap with a drop of dry sperm in the fertilizing tube was then put into place, the cap filled with water and the readings begun. The starting of the experiment and the making of the observations and recording them occupied the full time of two persons. Readings of the main thermopile, θ_a, were made every 60 seconds, and of the auxiliary thermopile, θ_b, every five minutes, for a period of from two and a half to three hours. Experiments were in only a few cases continued beyond the three hour limit. When steady conditions had been reached and enough readings had been taken so that the heat production of the unfertilized eggs could be determined, the pipette was lowered until its tip was below the surface of the egg suspension, the sperm washed out and stirred into the suspension, and the pipette raised again. This operation seldom caused an irregularity of more than 0.0005° C. At the same time a sample of the same batch of eggs was fertilized in a finger bowl, and kept surrounded by running sea water. These eggs were examined from time to time. The galvanometer zero and the storage cells for the potentiometer were checked frequently. At the end of the run the average diameter of the eggs was measured, in addition to the usual data on the percentage of fertilization and development. The volume of the single egg computed from the average value of the diameter, and the total volume of eggs

used was determined by centrifuging the suspension. After the centrifuging, the eggs were deformed so that their volume represented almost all of the volume measured; thus it was possible

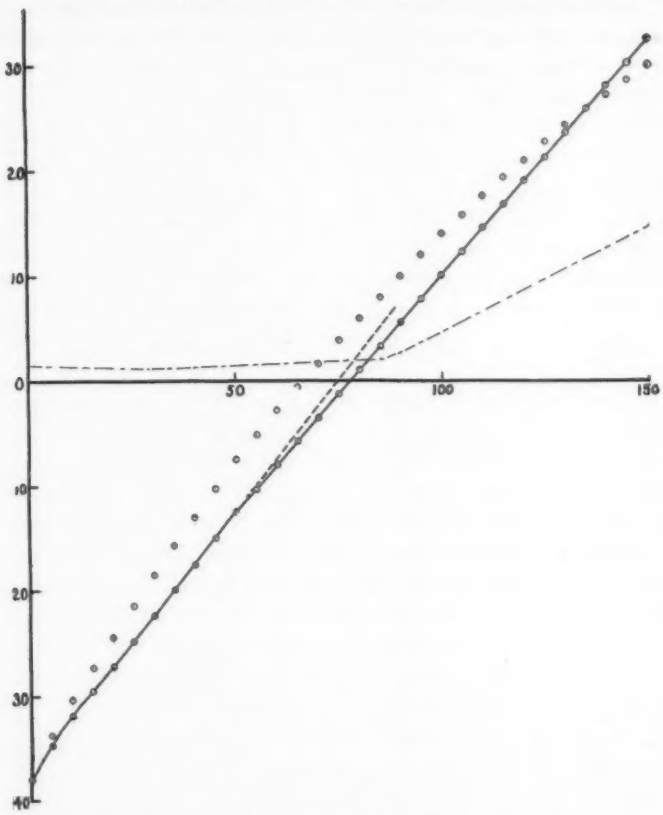

FIG. 3. Typical experimental curve. Heat production of *Arbacia* eggs. Abscissæ, time in minutes after fertilization. Ordinates, temperature differences in micro-volts. $1\mu v = 0.001364°$ C. ⊙ θ_a observed values. — — — θ_b observed values. —⊙— corrected curve. — — — projection of corrected curve of 20 to 50 minute period beyond the 50 minute position.

to obtain an estimate of the total number of eggs used. This method of counting was checked against a dilution method, similar to that employed for counting blood corpuscles, and it

was found that the agreement was very close, and that the eggs showed less variation than did the number of eggs in the fractions counted.

The course of a typical run is shown in Fig. 3. In it the average diameter of the eggs was 74 microns, and the total

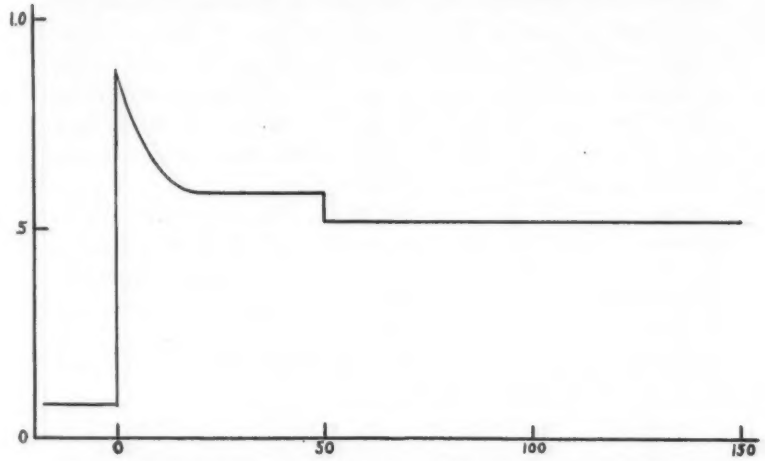

FIG. 4. Rate of heat production, *Arbacia* eggs. Abscissæ, time in minutes after fertilization. Ordinates, rate of heat production in calories per hour per million eggs.

volume was 0.82 cc. The total number of eggs was, therefore, about 3.9 millions. About 96 per cent. of these eggs were fertilized and of those fertilized about 85 per cent. were in the eight cell stage, and the rest in a late four cell stage and going into the eight cell stage so rapidly that an accurate percentage could not be obtained, when the experiment was concluded. In Fig. 3 every fifth reading is plotted. The corrected curve was obtained by taking approximate values of the integrals of equation (3) for five minutes intervals. The greatest variation in the different runs is in the behavior during the first twenty minutes after fertilization. This is probably due to the fact that the amount of sperm could not be made proportional to the number of eggs, and that the heat production of the sperm is not negligible during this period, as will be shown later. In this

respect the run shown in Fig. 3 shows a marked variation from the average.

The average of seven runs is shown in Fig. 4, which gives the approximate *rate* of heat production. The rate of heat production per million unfertilized eggs is about 0.08 calories per hour, while for the fertilized eggs after they have gone into the two cells stage it is about 0.52 calories per hour.[1] Both of these values are higher than those obtained by Shearer and by Myerhof with other sea urchins, but the ratio of fertilized to unfertilized eggs is the same. It should be pointed out that the results here given must be taken as indicative rather than conclusive and that further painstaking work is necessary.

One feature of the curve shown in Fig. 4 deserves special comment. It will be noted that the greatest rise in temperature, *i.e.*, the greatest period of heat production occurs immediately upon fertilization. This certainly raises again the question as to whether the process of membrane elevation depends upon an oxidative process, set up by the sperm cell when it comes into contact with the surface of the egg.

It must be mentioned here that Loeb (3) had expressed in 1906 the view that the essential feature (or possibly one of the essential features) of the process of fertilization is the increase in the rate of oxidation in the egg, and that this increase is caused by the membrane formation. Both Warburg and Loeb and Wasteneys had shown that the rate of oxidation in the sea-urchin egg is increased from 400 per cent. to 600 per cent. upon the entrance of the spermatozoön—and that membrane formation alone, induced by artificial means, has the same result. There is, therefore, a definite relation existing between membrane formation and increased rate of oxidation. From Warburg's (8) work it also seems likely that the increased oxidation occurs chiefly at the surface of the egg. The fact that the greatest heat production by the egg comes immediately after fertilization seems to us to make it plausible to say that the entrance of the spermatozoön induces a cortical oxidation process, and that this process results in the elevation of the fertilization membrane. The almost explosive character of the heat evolution seems to

[1] This involves an energy liberation of approximately 1 erg per egg per hour.

indicate that the oxidative process is of fundamental importance in the series of fertilization phenomena.

In the only run made upon sperm, ten drops of dry sperm were placed in the pipette and mixed with 50 cc. of sea water after steady conditions had been established. The heat produced can be expressed quite accurately by an equation of the form

$$H = H_0(1 - e^{-bt}),$$

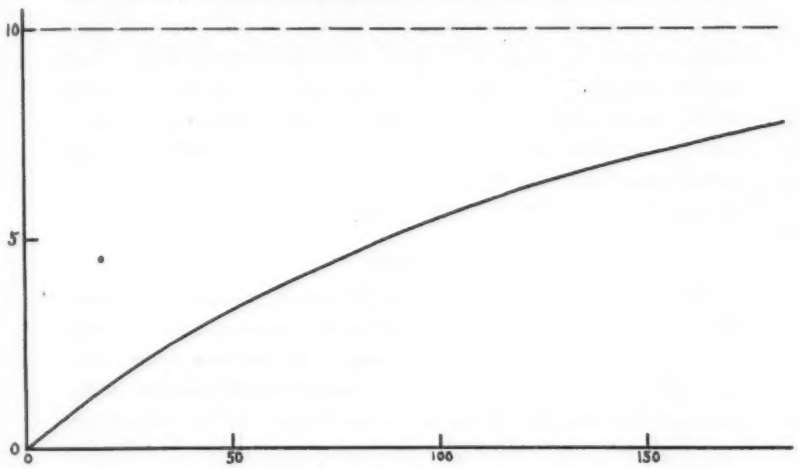

FIG. 5. Heat production of *Arbacia* sperm. Abscissæ, time in minutes after one drop of sperm is placed in 50 cc. sea water. Ordinates, temperature difference in micro-volts.

where H_0 is the total amount of heat produced—which, from the work of Cohn (1) is probably a constant for any given amount of sperm, b is probably dependent upon the pH of the water and the temperature, e is the natural base of logarithms, and t is the time after the sperm comes in contact with the water. This equation suggests very much the heat production by an exothermic reaction of the first order. In this case for ten drops of sperm,

$$H_0 = 0.79 \text{ calories},$$
$$b = 0.008 \text{ when } t \text{ is in minutes}.$$

Figure 5 shows the temperature change when one drop of sperm (approximately the amount used) is added to 50 cc. of

sea water. The curve approaches 10 micro-volts as an asymptote, but reaches 50 per cent. of that value in about 90 minutes. From this it will be seen that if there is an excess of sperm it may seriously affect results during the first twenty or thirty minutes.

As has been pointed out this method can not be used over long periods of time with the desired degree of accuracy. It also has the disadvantage of requiring large numbers of eggs, so that the longer they run, the more they tend to fall out of step, and so tend to mask any effect that may be present. This latter difficulty will remain, of course until it is possible to work with a single egg. The apparatus is being redesigned so that it will be possible to follow the heat production of both eggs and sperm under varying conditions over longer periods of time. It is planned to extend the work so as to include other forms.

Summary.

The heat production of the eggs of *Arbacia punctulata* has been measured before, during, and following fertilization, through development into the eight cell stage. It has been found that the rate of heat production at the instant of fertilization is ten to twelve times that of the unfertilized egg. After fertilization the rate of heat production decreases constantly for twenty minutes, when it reaches about 65 per cent. of the value at fertilization, and remains constant until the first cleavage, at about 50 minutes after fertilization. At the first cleavage the rate drops suddenly by more than 10 per cent., and then remains constant until the eggs are in the eight cell stage, which is as far as the work has been carried. The rate of heat production of the unfertilized eggs was found to be about 0.08 calories per hour per million eggs, and that of the fertilized eggs about 0.52 calories per hour per million eggs after the one cell stage.

An experiment on *Arbacia* sperm indicates that when placed in contact with sea water, its heat production is similar to that of an exothermic chemical reaction of the first order.

The suggestion is offered that the heat evolution occurring immediately upon fertilization is the result of an oxidative process which takes place in the cortex (chiefly) of the egg and which leads to the elevation of the fertilization membrane.

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CONCERNING THE RELATIVE PHOTOTROPISM OF VESTIGIAL AND WILD TYPE *DROSOPHILA*.

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In a previous investigation upon various phases of the light and gravity reactions of *Drosophila melanogaster*, the present writer concluded among other things, that the mutant race known as vestigial is much less phototropic than is the wild fly.[1] Subsequent to this, however, W. H. Cole, using a somewhat different technique from that of the author, arrived at quite another conclusion.[2] He made his tests in a vertically placed glass tube, with the source of light above it, and under these conditions he found that vestigial flies when attempting to climb the smooth walls of the tube constantly lost their footing, and slipped back. Thus they made poorer records than did winged insects, which, when they slipped, used their wings to catch themselves. This circumstance, according to Cole, entirely accounts for any apparent difference in the phototropic response of these two types of *Drosophila*. The correctness of this interpretation he seems to have proven by lining the glass with thin rice paper which enabled the wingless animals to obtain a secure footing, under which condition they made as good records as did the flies with wings.

It is to be noted that throughout these experiments only tests with a vertically placed tube were made. This was done on the assumption that the negative response to gravity which *Drosophila* gives would be constant, and that, therefore, any additional response would be due solely to the light placed above the tube. That this was apparently true Cole showed by testing the insects when only a very dim red light was present, as well as with lights of varying intensities. Nevertheless, in view of the

[1] McEwen, R. S., "The Reactions to Light and to Gravity in *Drosophila* and its Mutants," *Jour. Exp. Zoöl.*, 25: 49, February, 1918.

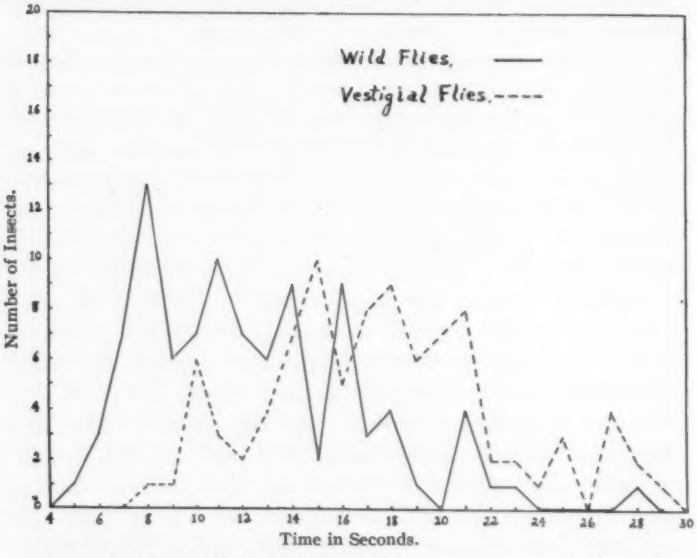
[2] Cole, W. H., "Note on the Relation Between the Photic Stimulus and the Rate of Locomotion in *Drosophila*." *Science*, LV., No. 1434, June 23, 1922.

author's previous results it seemed to him that either this mixing of the two responses, or perhaps some other unrecognized factor, might have produced complications which would account for the discrepancy in results. It was, therefore, determined to repeat Cole's work, using so far as possible his own technique, but with the addition of tests in horizontal tubes. The method employed was as follows:

A number of wild and vestigial flies were kept in bottles containing a plentiful supply of food in which larvæ were working, until five days after hatching. On the eve of the sixth day from ten to fifteen insects of each kind and of the same sex were removed, etherized, and each animal placed in a separate vial with a small amount of food. The next morning each insect was given a preliminary test within its vial, to determine so far as possible its general activity. This was done for the vestigials by turning the glass one end up and then the other four or five times. The five vestigial flies which responded most readily by giving the negative reaction to gravity, and which showed the greatest facility in crawling up the smooth glass of the vials, were then selected for the critical tests. Five wild flies were similarly chosen, except that their reaction to light was used as a criterion of activity, and an effort was made to pick those which reacted by crawling rather than by flight. In this connection it might be thought that the use of light rather than gravity as the stimulus for the winged flies would result in the selection of particularly phototropic animals. However, since virtually all wild type *Drosophila* are decidedly phototropic, the fact that those which only crawled to the light were chosen, would mean that if anything the less phototropic ones were selected. This preliminary work was then immediately followed by the critical tests of the ten flies in a manner to be described below. A similar procedure was continued on successive days until fifty wild males and fifty vestigial males had been tested; the same was then done with fifty females of each kind, making a total of 100 insects of each type. Each day's critical tests involved the following apparatus and manipulation:

Two tubes were used, one with no lining, and the other lined with thin rice paper throughout the portion through which the

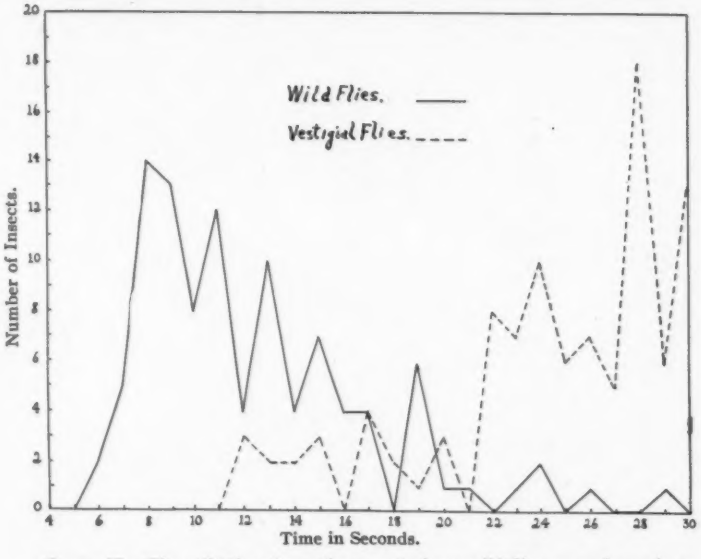
fly was to crawl. Each tube measured 16 mm. in internal diameter, and the length from the end away from the light to the point where the trial terminated was 172 mm., this being identical with the distance employed by Cole. Beyond this point the tube continued for 70 mm. to the end, which was



GRAPH I. The solid line shows the record, corrected for use of wings, of 95 wild flies, 45 males and 50 females, in an unlined vertical tube. The broken line, shows the record, corrected for slipping, of 92 vestigial flies, 45 males and 47 females also in an unlined vertical tube. In each case the time is plotted against the number of insects.

sealed with a piece of cover slip. A 75 watt gas filled lamp on a 110-volt alternating current was used as the source of illumination, and the distance from the end of the tube furthest from the light to the center of the concentrated filament of the lamp was approximately 317 mm. At the point from which the fly started, therefore, it was subject to an intensity of about 1,238 candle meters, an intensity slightly less than the maximum of 1,500 candle meters used by Cole. At the beginning of a test an insect was transferred from its vial to the testing tube by making use of its light or gravity reaction, or when necessary

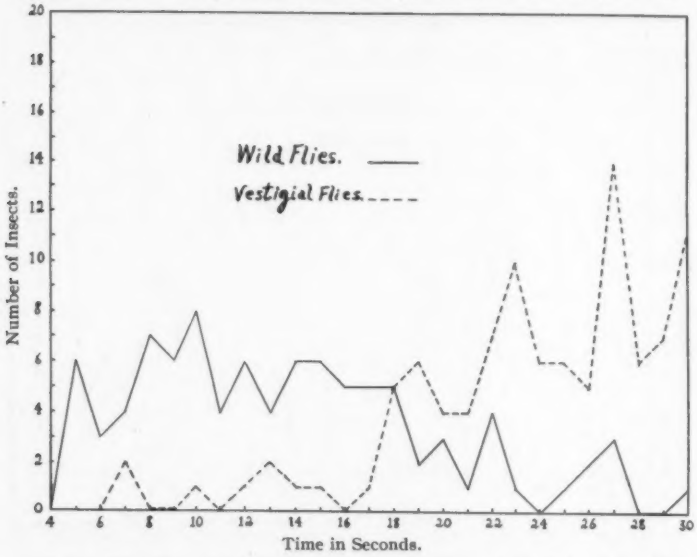
by shaking. The end of the tube was stopped by a cork covered with black paper, and the tube was placed in position. Ten successive trials were given in each test, the time of movement through the distance of 172 mm. being taken by a stop watch. If the passage was not accomplished within 30 seconds, a trial was considered completed, and 30 seconds was recorded as the



GRAPH II. The solid line shows the record of 100 wild flies, 50 males and 50 females, in a lined vertical tube. The broken line shows the record of 100 vestigial flies, 50 males and 50 females, also in a lined vertical tube. In each case the time is plotted against the number of insects.

time made. After each trial the animal was immediately returned to the starting point, if possible by its light or gravity reaction, but frequently by shaking. The latter procedure was made necessary in order that an approximately uniform period (about half a minute) might elapse between each trial. In this manner each of the five flies of a given type was tested in immediate succession, the same tube (either the one lined by paper or the one unlined) being used in the same position (either vertical or horizontal) for each insect. When the test of one group of flies was thus completed, the vials containing the insects were

set aside in a dim light, and the test of the other group begun. In this way each of the two groups were given during the day four tests, so spaced that the time elapsing between the successive tests of any given insect was approximately two hours. Each of the four tests was of course varied as regarded either the type of tube used or its position, the four possibilities involved being:



GRAPH III. The solid line shows the record, corrected for flight, of 93 wild flies, 43 males and 50 females, in an unlined horizontal tube. The broken line shows the record of 100 vestigial flies, 50 males and 50 females, also in an unlined horizontal tube. Only three instances of slipping were noted in the case of the vestigials, and as correction for these would have made no significant difference it is not shown.

the unlined tube horizontal, the unlined tube vertical, the lined tube horizontal, and the lined tube vertical. Furthermore, for the purpose of overcoming any biased effect which might result from always testing the same type of fly in the same condition with respect to time of day and previous tests, the order of the tests was rearranged each day. Under this plan during the course of the experiments each of the two types of insects was tested under almost (though not quite) every possible combination with regard to the above factors. The variations in the

arrangement of the tests thus obtained, though not entirely complete, were felt to be at least amply sufficient to avoid any significant effect from such a source of error. The results obtained by these methods are presented concretely in Table I., and Graphs I., II., and III.

TABLE I.

WILD FLIES.

Tube Horizontal.		Tube Vertical.	
Test I.,[1] Lined.	Test II., Unlined.	Test III., Lined.	Test IV., Unlined.
Male 12.6 Female 12.9 Combined Average 12.8 Probable Error .287	Male 10.5 Female 15.2 Combined Average 12.8 Probable Error .341	Male 11.7 Female 13.9 Combined Average 12.8 Probable Error .31	Male 10.8 Female 14.2 Combined Average 12.5 Probable Error .29
	Corrected for Flight Male 12.1 Female 16 Combined Average 14.2 Probable Error .425		Corrected for Flight Male 11 Female 14.3 Combined Average 12.7 Probable Error .308

VESTIGIAL FLIES.

Tube Horizontal.		Tube Vertical.	
Test I.,[1] Lined.	Test II., Unlined.	Test III., Lined.	Test IV., Unlined.
Male 24.7 Female 26.1 Combined Average 25.4 Probable Error .25	Male 22.9 Female 25.2 Combined Average 24 Probable Error .335	Male 24 Female 25 Combined Average 24.6 Probable Error .325	Male 18.5 Female 19.1 Combined Average 18.8 Probable Error .348
			Corrected for Slipping Male 17.4 Female 18.5 Combined Average 18 Probable Error .337

[1] These numbers are merely for convenience in designating, and do not indicate that the tests were always given in this order (see text).

As regards the mathematical procedures used in obtaining the values shown in the table and graphs the following should be said: The record of each fly in any given test was secured by taking the average of its ten trials in that test. The 100 records of all the individual insects of one type (winged or vestigial) were then in turn averaged for each of the four tests indicated. Both in making the graphs and in computing the probable errors the records of the 100 flies obtained as just described were employed, rather than the results of all their individual trials. This was the essential procedure throughout, but the details were slightly modified in certain cases for these reasons. It was realized shortly after the beginning of the experiments that in view of the question at issue it would be highly desirable to have for comparison averages which had been corrected, so far as possible, for the factor of flight in the case of the winged flies, and for slipping upon the unlined glass in the case of the vestigials. The modifications thus made necessary in the case of the winged animals in the unlined horizontal tube were as follows: Since 7 insects had been tested before observations on this point were begun, the records of these 7 had to be thrown out entirely. In the cases of the remaining 93 flies, wherever necessary new records were computed for each animal by eliminating any of its ten trials in which use of the wings in any manner had occurred. This process involved, as it turned out, 53 insects, with a total of 157 trials, which is an average of 2.9 trials per insect concerned; the highest number which any one animal thus lost was 7. The new records of these 53 flies were then averaged in with the 40 which required no change to obtain the so-called "corrected for flight" average. In the case of the winged flies in the vertical tube, correction was made in the same manner, except that in this case only the records of the first five animals had to be entirely discarded because observation on this point did not begin until after they were tested. In this instance out of the remaining 95 flies the records of 40 had to be corrected, involving 70 trials, an average of 1.75 trials per fly concerned. Again the highest loss suffered by any one animal was 7. The third correction was that for the slipping of vestigial flies in the vertical unlined tube. The same general method was employed as in

the case of flight, a total of 16 animals being affected. Of these the records of 8 had to be eliminated entirely because no effort had been made to distinguish their behavior in individual trials. To the 80 trials thus counted out had to be added 11 trials distributed among the other 8 flies, the highest number lost by any one of the 8 being 3. Thus a total of 92 insects were tested, the records of 8 of them being corrected by the elimination of the trials in which slipping occurred.

It will be noted of course that the probable error in all the determinations is quite high, as the character of the curves would lead one to expect. This is due to the usual erratic behavior of *Drosophila*, which the writer has always found characteristic of this animal. Nevertheless, in view of the very marked differences between the corresponding records of wild and vestigial insects, it is felt that the results are certainly significant. Thus it may be noted that even when the probable errors are multiplied by five, the ranges so obtained do not overlap, except in a single instance. This instance is that of the range for wild flies in the horizontal unlined tube corrected for use of wings, and the range for vestigials in the vertical unlined tube corrected for slipping. Here the upper value for the range of the wild flies just equals the lower value for the range of the vestigials.

The conclusions indicated in the table and graphs may now be summarized thus: (1) The vestigials under every condition are decidedly slower than the wild type; (2) the writer's work fails entirely to confirm Cole's contention that the slipping of the vestigials is responsible for their slower records.

These conclusions come out even more clearly when the data are analyzed in more detail. Thus not only does correction for use of wings by the wild flies make no significant difference, either in the horizontal or vertical records, but correction for slipping in the case of the vestigials likewise produces essentially no effect. Curiously enough indeed, it appears both from the table and the graphs that the vestigials did much better in the vertical tests when the tube was *unlined*, while no very marked difference occurs between their records for the lined and unlined tubes in the horizontal tests. In the case of the wild flies, on

the other hand, there is no significant difference between the records for the lined and unlined tubes in either the horizontal or vertical tests. In this connection it might further be stated that not only does the data based upon timing indicate that slipping is not a significant factor in retarding the vestigials, but that observation of their actual behavior leads to the same conclusion. Thus in the vast majority of cases the writer was unable to see that the vestigial insects had the slightest difficulty in crawling upon the unlined glass. Regarding the matter from yet another aspect it is instructive to note the records for corresponding groups where the only difference involved is the position of the tube. Here it appears that in the case of the wild flies in the unlined tubes the vertical position is slightly superior both as regards the arithmetical average and the mode. In the lined tubes on the other hand, only the modal value for the vertical position is superior. As regards the vestigials, the lined tubes again show only a slight superiority for the vertical records but in the case of the unlined tubes the vertical position is markedly superior, both as to average and mode, *even without the correction for slipping*. This of course is exactly opposite to what one would expect according to Cole's results and interpretation.

Besides this analysis of the data as a whole, it is also pertinent to call attention to certain facts brought out by observation of individual insects. Thus in the case of the horizontal tests of vestigial flies in the unlined tubes, where the behavior of the animals could be perfectly observed, the following feature was noteworthy. There were 14 insects which were noted on the records, not only as having no tendency to slip, but as being unusually active; *i.e.*, they crawled rapidly and constantly about. Yet the average time for these 14 cases was 23.5 seconds, virtually the same as that of the general average for the test. There were likewise 16 vestigials in this test which were recorded as "quite" active, with an average of 24 seconds, and 12 recorded as "fairly" active with an average of 27.4 seconds. Hence the mere activity of these animals does not seem to have materially affected their tendency to move toward the light. The only feasible explanation of such cases would seem to be that such flies are only weakly phototropic. This interpretation is given

further emphasis by one other observation. It was noted in the horizontal tests of vestigial animals that a fly which had reached the light end of the tube would often turn immediately about and crawl quite as rapidly all the way to the dark end. Such a performance, though it would appear very favorably on the record, would obviously not denote any great degree of phototropism. This occurred, however, in at least one of the trials of each of 19 individuals. On the other hand, such action was noted in only two trials of wild type animals. In the case of vertical tests it never occurred with vestigials, but one of the two instances of wild flies took place in such a test. The reason for this single instance is not clear, and in the present state of our knowledge can only be ascribed to the erratic tendency already noted in *Drosophila*. In a similar category, perhaps, must be put the behavior of 9 vestigial insects which, though recorded as active, made relatively poor records in the vertical tests.

How the results and conclusions indicated by Cole can be reconciled with those herein presented it is rather difficult to see. In any event it is perhaps worth while summing up certain outstanding points which the two sets of experiments seem to bring out. In the first place it does not appear that the mixing of the light and gravity reactions, which was suggested as a possible source of error, has any effect upon the main question at issue. Secondly, though also apparently without bearing upon this question, is the fact that Cole's wild type flies in vertical tubes seem to have been much faster than those of the writer. With 1,500 candle meters 50 per cent. of Cole's animals averaged 6.17 seconds and with 750 candle meters 7.6 seconds. Upon the other hand, the best average obtained by the writer was 10.5 seconds, the remainder of them being from two to four seconds slower, under a stimulus of 1,238 candle meters. Such a discrepancy almost causes one to wonder whether there may not have been some fundamental difference in the character of the insects used or in the experimental conditions. Thus, for example, the tests herein recorded were conducted at an average temperature of 24.1 degrees centigrade, and it is known in a general way that the activity of *Drosophila* varies more or less

directly with this factor. Unfortunately Cole gives no record of temperature. Nevertheless, so far as data on this point go, it seems improbable that his work was done at a sufficiently higher temperature to account for the difference in results. In this connection another feature of Cole's work which makes for some uncertainty is the fact that the records for his flies just cited apparently refer to only 50 per cent. of the insects tested. What the other 50 per cent. did might possibly be significant, at least as regards comparative records for the wild type. Lastly, perhaps the most pertinent point to be indicated is this. One conceivable explanation for the poor showing of Cole's vestigial animals in the unlined tube is the fact that presumably they were not previously selected for their ability to crawl on glass, as was the case to a certain extent with those used by the writer. It seems clear, however, that neither this, nor any of the other points mentioned, account at all for the fact that whereas Cole's results showed the vestigials, when given a proper footing, nearly or quite the equals of his winged flies, in the present experiments the wild type completely outclasses the vestigials under all conditions, even when correction has been made for use of wings by the former and slipping by the latter.

THYROID FEEDING AND SECONDARY SEX CHARACTERS IN RHODE ISLAND RED CHICKS.

HARRY BEAL TORREY AND BENJAMIN HORNING.

In an earlier paper [1] we called attention to the precocious appearance of the second set of rectrices in Brown Leghorn chicks whose daily ration from the fourth week after hatching had included dessicated thyroid. Reference was made also to another case of precocious development of rectrices, namely in Rhode Island Red male chicks,[2] as a result of thyroid feeding. And, correlated with the early appearance of the rectrices in the latter was a failure of the feathers on the neck to differentiate into the hackles characteristic of the control males. The acceleration of rectrices and absence of hackles of the male type combined to give to the plumage of the thyroid-fed males an aspect so strikingly female as to deceive even experienced poultry fanciers. Comb, wattles, carriage of the body and behavior remained, however, characteristically male. The accompanying tables present typical facts and the figures typical birds.

Table I. shows the effect of thyroid feeding in one experiment on the development of the first rectrices in Rhode Island Red chicks. When the latter were four weeks old, they were divided into two lots as indicated in the table. To each bird of one lot a capsule of Armour's desiccated thyroid, containing .2 per cent. I, was given daily. For the first two weeks the dose was 50 mgms.; for the next three weeks, 100 mgms.; for the next three weeks, 150 mgms. The other lot served as a control. All birds were twelve weeks old when recorded.

Figure 1 is from a photograph of the typical bird recorded as No. 1, Table I., at the age of twelve weeks. The rumpless condition is characteristic of normal Rhode Island Red males at this age. It is due to the fact that the first set of rectrices customarily

[1] Torrey and Horning, The Effect of Thyroid Feeding on the Moulting Process and Feather Structure of the Domestic Fowl, BIOL. BULL., XLIX, 1925, 275.

[2] Torrey and Horning, Hen Feathering Induced in the Male Fowl by Feeding Thyroid, *Proc. Soc. Exper. Biol. and Med.*, XIX., 1922, 275.

TABLE I.

Control Birds.				Thyroid-fed Birds.			
No.	Sex.	Rectrices.	Type.	No.	Sex.	Rectrices.	Type.
1	Male	0	Male	11	Male	+	Female
2	Male	0	Male	12	Male	+	Female
3	Female	+	Female	13	Female	+	Female
4	Female	+	Female	14	Male	+	Female
5	Male	0	Male	15	Male	+	Female
6	Male	0	Male	16	Male	+	Female
7	Female	+	Female	17	Female	+	Female
8	Female	+	Female	18	?	+	Female
9	Female	+	Female	19	Female	+	Female
10	Male	0	Male	20	Female	+	Female

A plus sign indicates the presence of rectrices.

FIG. 1. Normal R. I. R. male (No. 1, Table I.) twelve weeks old. The absence of rectrices is typical at this age.

does not appear. There are exceptions to this rule, but in these cases, the rectrices are small and more or less atypical. There is some variability in the form of the first rectrices in the female, the stoutness and placement of the feathers and the shape of the feather tips being most frequently affected, less often the number. But whatever their form, the rectrices are characteristically absent in the male, present in the female.

FIG. 2. R. I. R. male (No. 12 of Table I.) twelve weeks old, thyroid fed for eight weeks. Rectrices well developed.

In thyroid-fed males, they are characteristically present also, appearing about the time they appear in normal females. Fig. 2 is from a photograph of No. 12, Table I., twelve weeks old. Fig. 3 is another thyroid-fed bird, aged twelve weeks, being No. 15, Table I. In each case the head is typically male, the feathering typically female. Comparison of Figs. 2 and 3 with Figs. 4 and 5

will emphasize these facts. Fig. 4 is from a photograph of No. 4 Table I, a typical control female twelve weeks old. Fig. 5 is from a photograph of No. 13, Table I, a typical thyroid-fed female of the same age.

FIG. 3. R. I. R. male (No. 15 of Table I.) twelve weeks old, thyroid fed for eight weeks. Rectrices well developed.

There was no essential difference between the last two birds at the time the photographs were taken. The conclusion, however, that thyroid feeding does not affect the female plumage at this age must not be hastily drawn. When thyroid-fed chicks are compared with controls at an earlier age, evidence is obtained to show that thyroid feeding accelerates the development of both rectrices and contour feathers in females as well as males.

In Table II. such evidence is summarized. The chicks were 51 days old. Eight days after hatching, thyroid feeding had been begun with 15 mgms. as the initial daily ration, in capsule as

before. This had been increased from time to time, the chicks being fed as much thyroid as they could stand without displaying obvious signs of weakness or distress.

TABLE II.

Control Birds.				Thyroid-fed Birds.			
No.	Sex.	Rectrices.	Contours.	No.	Sex.	Rectrices.	Contours.
65	Female	1 cm.	Not well out	81	Female	?	Not well out
66	Female	0	" " "	82	Female	7.5 cm.	Well out
67	Female	0	" " "	83	Female	2.5 cm.	" "
68	Female	0	" " "	84	Female	1 cm.	" "
69	Female	0	" " "	85	Female	5 cm.	" "
70	Female	0	" " "	86	Female	2.5 cm.	" "
71	Female	0	" " "	87	Female	2.5 cm.	" "
72	Female	0	" " "	88	Female	0	" "
73	Male	0	" " "	89	Male	0	Not well out
74	Male	0	" " "	90	Male	2.5 cm.	Fairly well out
75	Male	0	" " "	91	Male	1 cm.	Well out
76	Male	0	" " "	92	Male	1 cm.	" "
77	Male	0	" " "	93	Male	4 cm.	" "
78	Male	0	" " "	94	Male	2.5 cm.	Not well out
79	Male	0	" " "	95	Male	4.5 cm.	Well out
80	Male	0	" " "	96	Male	4.5 cm.	" "
				97	Male	0	Not well out

It will be seen on inspection of Table II, that there is considerable variation in the length of the rectrices and the condition of the contour feathers in thyroid-fed birds of both sexes. There are differences, not here recorded, in size and weight also, that are not, however, strictly correlated with differences in the feathering.

FIG. 4. Normal R. I. R. female (No. 4, Table I.) twelve weeks old.

We are aware that such variability is a source of difficulty in the interpretation of results. It is greatest in the first few weeks after hatching, when it is attributable partly to irregularities in nutrition and to differences of vigor referable to other causes. These tend to disappear with age. But the constitutional variability of the Rhode Island Red breed, which we have learned to recognize in the course of our work, remains as a source of error. All of our experiments have been checked in other breeds, notably in Leghorns (Torrey and Horning, 1925) and Campines, where

FIG. 5. R. I. R. female (No. 13, Table I.) twelve weeks old; thyroid fed for eight weeks.

the forcing effect of thyroid feeding on the development of the plumage is clear and definite. In the latter breeds, however, the first rectrices normally develop simultaneously in both sexes.

The variability of Rhode Island Reds led us formerly into a conclusion that proved to be erroneous. In the first series of experiments reported, our five thyroid-fed capons failed to develop rectrices like unaltered thyroid-fed males. Later it was discovered that this result was exceptional. The gonad had, in the male, no bearing on the result.

This misconception led us to suspect that the effect of thyroid feeding on the feathering of the male might be indirect, through an augmentation of the so-called luteal tissue, to whose presence in the testes of Campines and Sebright bantams has been imputed the hen-feathered condition of the males of these breeds. No support for this suspicion was obtained from histological preparations, however, and the observation of the thyroid effect in capons completely disposed of the possibility of it. But that the thyroid effect is not independent of the ovary in certain respects is a fact that will be developed in another connection.

The intensity of hen feathering in males of hen-feathered breeds differs with the breed. For instance, the male silver Sebright bantam is an absolute replica of the female, in plumage. The male silver Campine, however, though resembling the female closely, differs from it not only in the possession of sickle feathers but in the structure of the hackles, which are laced in the typical male fashion.

Now the plumage of thyroid-fed Rhode Island Red males, up to the age of 12 weeks, is indistinguishable from that of females. Later on, the males in our experiments developed plumage that is perhaps best described by saying that it was prevailingly male with certain modifications characteristic of the female type.

In place of typical male hackles (Fig. 6, *a*, *b*) with broad marginal lacing of naked barbs, and with sharply pointed tips, thyroid feeding produced hackles with broader and more rounded or truncated tips, and narrower lacing, especially toward the tips, but, more or less irregularly, elsewhere also (*c*, *d*). Saddle feathers (Fig. 7, *a*, *c*) were similarly affected, the ends being broader and more rounded, and the laced margin furnished with an irregular inner border owing to the development of barbules at points where the barbs are normally naked (Fig. 7, *b*) or in exceptional cases, to the absence of barbules in normal situations. We shall have occasion to discuss these irregularities elsewhere.

The characteristically broad distal lacing of shoulder feathers in normal males (Fig. 8, *a*) was still more strikingly reduced in thyroid-fed males (*b*), often, in fact, completely obliterated (*c*), giving the feathers an undeniably female aspect, as can readily be seen by comparing (*c*) with (*d*), the latter being a feather from the shoulder of a normal female.

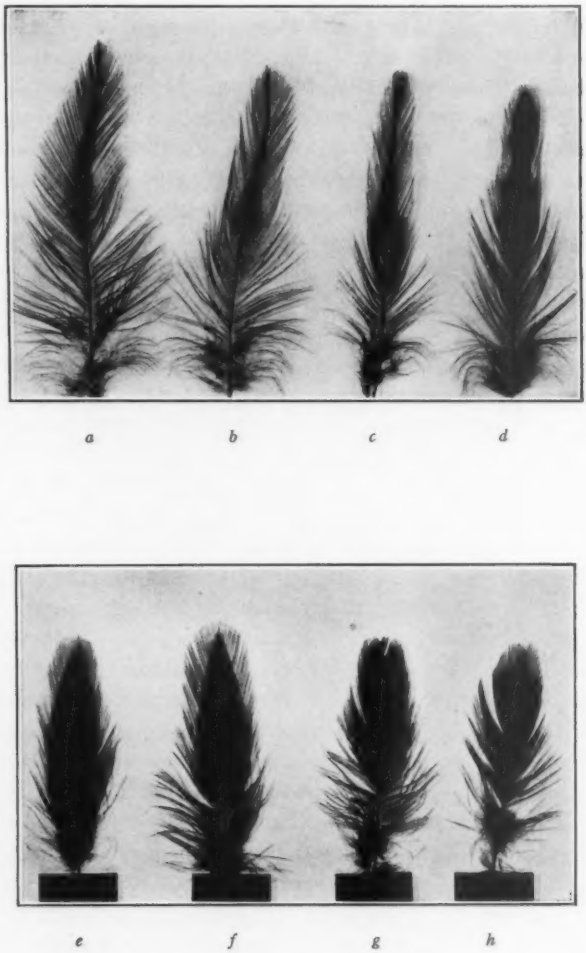


FIG. 6. Hackles from R. I. R. birds about seven months old: *a*, *b*, from a normal male; *c*, *d*, from a thyroid-fed male; *e*, *f*, from a normal female; *g*, *h*, from a thyroid-fed female.

To a less but still striking degree, the hackles of the thyroid-fed males tended to assume the aspect of female hackles. Compare, with this in mind, Fig. 6, *a*, *b*, with Fig. 6, *e*, *f*, *g*, *h*. And note also that, in this series of feathers, the hackles of the female, which are here exceptionally male in structure, are modified in the same direction.

To a still less degree than the hackles do the saddle feathers of thyroid-fed males approximate the saddles of normal females: yet here too the changed shape of the feather and the added barbules and consequent diminished lacing suggest the female type.

a *b* *c*

FIG. 7. Saddle feathers from R. I. R. birds about seven months old: *b*, from a normal male; *a*, *c*, from a thyroid-fed male.

By way of summary it may be said that when male Rhode Island Red chicks were fed desiccated thyroid in daily doses increasing with their weight, two general effects were observed:

1. Their plumage appeared precociously but differentiated later than usual, so that at the age of 12 weeks they were feathered like females. It was to this effect that our earlier observations referred.

2. Their adult plumage, though prevailingly male in type, exhibited characteristics of form and structure, especially in the

hackle, saddle, back and shoulder feathers, that are found ordinarily only in the corresponding feathers of females.[1]

And it may further be said that when the lacing characteristic of the male hackle appears also in the female that the effect of thyroid is to modify it also, as in the male, by the addition of barbules.

a *b* *c* *d*

FIG. 8. Shoulder feathers from R. I. R. birds about seven months old: *a*, from a normal male; *b*, *c*, from a thyroid-fed male; *d*, from a normal female.

Thyroid feeding, then, tends to produce hen feathering in the Rhode Island Red male, and, to the extent noted in the last paragraph, in the female also. Toward this result there is no coöperation of the gonad. That the ovary does influence the action of thyroid with reference to certain characters not, however, correlated with sex, will be shown in another paper.

ZOÖLOGICAL LABORATORY,
UNIVERSITY OF OREGON,
April 7, 1925.

[1] Crew and Huxley (*Veter. Jour.*, LXXIX., No. 10) appear to have seen neither of these effects in their thyroid-fed fowls. The first naturally escaped them, for their birds were too old at the beginning of the experiment to develop it. That the second effect also escaped them may perhaps be attributed to their material and methods. Their 12 birds, of which 6 were males, were F_1 hybrids from a cross between a Rhode Island Red ♂ and a Light Sussex ♀, and inherited the black hackles and white ground color from the mother. An amount of thyroid equal to 2 grains per bird was mixed every day into the common ration of wet mash. This dosage was smaller than ours, was not increased as the weight of the birds increased, and was so administered as to leave in doubt just how much each male obtained

STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES. IV. MALES WITH TWO SETS OF SUPERNUMERARY MALE CHARACTERS.

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In study number three of this series, males of *Cambarus propinquus* and of *Cambarus virilis* which bore supernumerary male characters were described. These supernumerary characters were in the form of copulatory hooks ordinarily found only upon the third walking legs but in these cases present also upon the second or the fourth walking legs. In *C. virilis* the extra hooks were confined to the second walking legs. In *C. propinquus* the additional hooks were found upon either the second or the fourth walking legs. It was predicted that in *C. propinquus*, since either the second or the fourth walking legs might function in carrying the extra hooks, specimens might be found in which copulatory hooks would be present upon both the second and the fourth walking legs.

During the summer of 1924 large collections were made from practically all the streams and some of the lakes of southeastern

FIG. 1. Diagram illustrating the arrangement of the copulatory hooks upon the third walking legs of a normal male specimen.

Wisconsin and in one stream three males of *C. propinquus* were found which bore supernumerary copulatory hooks upon one or both of the second and fourth walking legs.

DESCRIPTION OF SPECIMENS.

Specimen number one (Fig. 2) is a male of the first form and is 52 mm. long. Normal copulatory hooks are present upon the

third walking legs. Hooks a little smaller than those upon the third walking legs are present upon the right second and the right fourth walking legs. The left second and the left fourth legs possess no hooks.

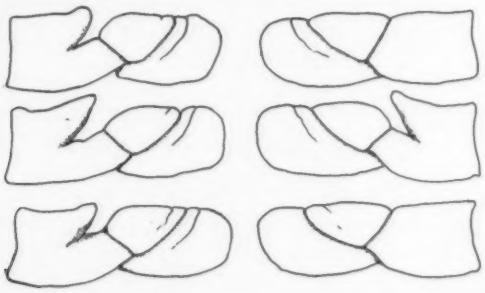

FIG. 2. Diagram illustrating the copulatory hooks upon the second, third and fourth legs as they occur in specimen No. 1.

Specimen number two (Fig. 3), also a first form male, is 43 mm. long and in addition to the usual hooks upon the third legs there are hooks also upon both of the second walking legs and

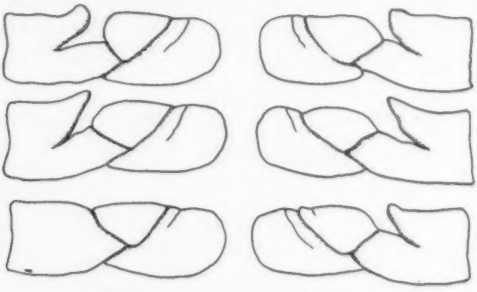

FIG. 3. Diagram illustrating the arrangement of the copulatory hooks upon the second, third and fourth walking legs in specimen No. 2.

upon the left fourth leg. The supernumerary hooks are again distinct but smaller than the normal ones upon the third walking legs.

Specimen number three (Fig. 4) is 47 mm. long and has copulatory hooks upon both of the second, third and fourth walking legs. The hooks upon the second and upon the fourth legs are slightly smaller than the normal hooks.

Frequency of Occurrence and Distribution.

The three specimens described here were the only ones taken among approximately 3,600 males of *Cambarus propinquus*. The condition may, therefore, be considered rare.

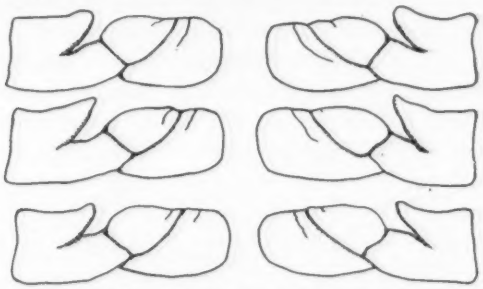

Fig. 4. Diagram illustrating three full pairs of copulatory hooks as they occur upon the second, third and fourth walking legs of specimen No. 3.

All three specimens were taken in a single section of one stream (Pike River near Racine, Wisconsin) and constituted about three per cent. of the males of the entire catch. Thirty-six other streams and rivers examined contained no aberrant specimens of this type.

Male specimens with one pair of supernumerary hooks are sometimes fairly abundant, in some localities representing as high a proportion as seven or eight per cent. of all the males. In Pike River, however, where the three specimens with two supernumerary pairs of hooks were taken, fifteen per cent of the males had one supernumerary pair of hooks, some of them bearing the extra hooks upon the second and others upon the fourth walking legs.

Discussion.

Some direct and considerable indirect evidence has been accumulated indicating that in female crayfishes with male secondary sexual characters the condition is inherited and should be considered a germinal mutation. It is significant in this connection that all three specimens of the males with two pairs of supernumerary hooks should come from the same locality. It is also suggestive that in this locality there should also occur by far the largest proportion of males with one supernumerary pair of

hooks. If it be assumed that the condition represented by the possession of supernumerary hooks upon the second legs is independent of the condition in which supernumerary hooks occur upon the fourth legs and that both conditions are inherited then it would follow that a combination of the two characters would be most reasonably expected in that locality where both were most abundant. Or, if it be assumed that there is present a cumulative, heritable factor which tends to produce one supernumerary character in the simplex state and added supernumerary characters when doubled then the locality in which doubling would be expected would be that locality in which the highest proportion of single supernumerary characters appeared.

This evidence for the inheritance of the characters described is not convincing in itself but it is important in that it supports the interpretation tentatively advanced for the occurrence of like aberrant structures found in females.

STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES. V. MALES WITH FEMALE CHARACTERS.

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A number of crayfishes belonging to the genus *Cambarus* in which secondary sexual characters of both sexes were found upon the same individual have been described by Faxon, Hay, Ortman and the writer. All of these specimens with the exception of one described by Hay were females. In some cases there was a full complement of female secondary sexual characters with the addition of one or more male characters and in other cases a female character was lacking while one or more male characters were present. The one exceptional specimen described by Hay was hermaphroditic and possessed both male and female primary and secondary sexual structures.

Male crayfishes in the genus *Cambarus* bearing sexual characters of females have not been noted before and it is the purpose of this paper to describe two specimens which appeared in recent collections made in southeastern Wisconsin. Both specimens were collected from tributaries of the Rock River near Beloit, Wisconsin, the first in July, 1923, and the second in July, 1924.

One is a specimen of *Cambarus virilis*, 65 mm. long and is a second form male normal in every respect except that an oviducal opening exists upon the left third walking leg. An examination of the internal organs shows a normal spermary and normal spermatic ducts. There is no tube of any kind joined to the oviducal opening at the base of the third walking leg. The second specimen is one of *C. propinquus*, 46 mm. long, and is identical in its peculiarities with the first specimen described. It is a normal second form male with all the normal internal and external structures of the male but bearing in addition an oviducal opening at the base of the third walking leg. As in the

previously described specimen there is no duct attached to the aberrant oviducal opening.

Discussion.

Since all the specimens taken previously which bore secondary sexual characters of both sexes in the same individual have been females or hermaphrodites the impression might easily arise that some mechanism exists which is capable of producing male secondary sexual characters in females but that either the same or some other mechanism prohibits the reciprocal production of female characters in males. Indeed, the following statement with this implication is made by Hay in a discussion upon "Hermaphroditism in Crayfishes."[1] "It would appear, therefore, that in the genus *Cambarus* at least hermaphroditic individuals are females which, owing to some ambiguity of the formative cells in the embryo, have developed to a greater or less degree characters of the opposite sex." The finding of only two males having each a single female character furnishes extremely meager data upon which to generalize but it seems important to demonstrate that crayfishes having secondary sexual characters of both sexes may sometimes be males and that no influence exists in the male which completely inhibits the development of female characters.

[1] Smithsonian Miscellaneous Collections. Sept. 8, 1905.

PHYSIOLOGICAL STUDIES ON HIBERNATION IN THE POTATO BEETLE, *LEPTINOTARSA DECEMLINEATA* SAY.[1]

DAVID E. FINK.

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INTRODUCTION.

An inquiry into the nature and cause of the phenomenon of hibernation has for many years engaged the attention of investigators. The literature on the physiological study of hibernating mammals covers a wide range of research, the most significant of which appears to be into the effects of temperature, food and gaseous exchange. The experimental evidence brought forward by Dubois (10), Pembrey (17), Valentin (25), Weinland and Riehl (29) and others, indicates that hibernating animals placed

[1] A thesis in zoölogy presented to the Faculty of the Graduate School of the University of Pennsylvania in partial fulfillment of the requirement for the degree of Doctor of Philosophy.

in the presence of an abundance of food and warmth remain impassive. This has led to the conclusion that hibernation is some inherent, deep seated phenomenon. Recent studies by Carlier (8), Rasmussen (18), Sheldon (22) and others, upon the nature and function of the so-called hibernating gland, show it widely present in mammals.

The literature on physiological studies of hibernating insects is not so extensive. Baumberger (1), Bodine (3), Breitenbecher (6), Roubaud (19), Sanderson (20), Tower (24) and others, however, have investigated problems of temperature, moisture, and gaseous exchange as they relate to insects. According to Baumberger (2), hibernation has resulted from the repeated effect of winter upon the species and the rhythmical phenomenon has been determined by the habits of the insect. Roubaud (19) recently advanced the view that a lowered metabolic activity permits a progressive physiological purification. According to this author, two factors, *anhydrobiosis*, and *athermobiosis* (dehydration and absence of heat), in themselves inhibitory, become the unexpected factors of reactivation by favoring the processes of excretion during dormancy.

Most investigators whether dealing with hibernation in vertebrates or invertebrates agree that the influence of temperature in the process is subordinated, and that hibernation is preceded usually by a period of extensive feeding and a consequent reduced metabolic activity. It is also well known that food is an important factor in influencing the habits of animals. The manifold instances of food storage indicate a varying adaptability, the most significant being the storage within the body of the animal of reserve nutrients in the form of fat.

With insects the phenomenon of hibernation is unique, because it may occur in any stage of the life cycle of different insects, from the egg to the adult. It seemed advisable, therefore, to investigate the problem of nutrient storage in insects and to determine the specific mode of its utilization during the progress of hibernation.

This contribution presents the results obtained from physiological studies on the potato beetle, *Leptinotarsa decemlineata* Say, before, during, and after hibernation.

The author desires to express his appreciation to Prof. C. E. McClung, and other members of the Zoölogical Department of the University of Pennsylvania, for generous aid and suggestions. To Doctor J. H. Bodine he is deeply indebted for helpful criticism during the progress of the work and for advising investigation of this problem.

Material and Methods.

The adult potato beetle, *Leptinotarsa decemlineata* Say, used exclusively in these investigations, was reared from eggs deposited by overwintering adults.

The methods adopted for different lines of investigation varied. The type of cage for determining the effect of various food plants consisted of ordinary six-inch flower pots filled with soil, in the center of which was sunk a 5 x 1 inch glass vial containing water and fresh food plant. The covering to this cage was made of mosquito wire in the form of a circular cage of a diameter to fit snugly within the inner upper rim of the flower pot, and varying in height from 12 to 18 inches. These cages were set in troughs of soil in an outdoor breeding house. A more elaborate cage consisted of a wooden framework (20 x 18 x 14 inches), constructed of inch pine, enclosed with mosquito wire and set on

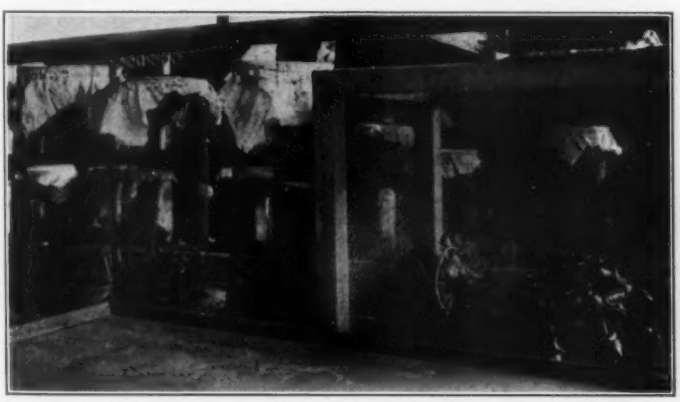

Fig. 1. Types of cages used in the food experiments. This figure shows two types: (1) circular cages composed of flower pots sunk in the soil with mosquito wire tops; (2) large oblong cages constructed of inch pine, covered with mosquito wire and set on the surface of the soil. Foliage was kept fresh in the cages by inserting them in vials of water.

the surface of the soil in the breeding house or in the field (Fig. 1). In all cages it was impossible for beetles to escape or for others to find entrance from the exterior. Access for the worker was by a convenient opening on the top or side of the cage.

The water content was obtained by first weighing and slitting the animals and afterwards placing them in an oven at a temperature between 90–95° C., until a constant weight was obtained. The percentage of water was computed on the basis of dry weight.

Although the extraction of fat from the body of an organism with solvents cannot be achieved quantitatively without change in the nature of the compounds in which the fatty acids are present, this was not considered an objection in these experiments as the result desired was the total fat content in whatever form it might occur. The tissues, however, had to be dried and powdered. To prevent partial oxidation of the more unstable unsaturated acids, the drying took place at room temperature, or in an oven at a temperature not exceeding 30° C. The extracting solvent used extensively was a mixture of equal proportions of ether and alcohol, which was warmed and renewed several times by decantation. In addition, the method described by Voltz (28), and modified to suit the material under investigation was also used. Calculations were made in percentage based on dry weight of the organism.

Metabolism determinations (respiratory exchange) were obtained by methods essentially similar to those described by the author (12) in a previous publication. Other methods used will be described in the text under appropriate headings.

Experiments and Discussion.

Food Experiments.

The effect of different food plants on potato beetles of the first generation emerging from pupation was studied. Altogether, during June, 1923, and 1924, about 800 beetles were divided into four main groups and fed on various food plants as follows:

Group 1. The foliage of potato, tomato, eggplant and peppers.
Group 2. The tubers or fruit of the above plants.
Group 3. The foliage of potato for six days, followed by sliced

non-solanaceous foods like beets, carrots, squash and cucumbers.

Group 4. Non-solanaceous foods exclusively.

Number of Days Feeding Before[1] *Hibernation.*

The number of days feeding on different food plants before hibernation took place is shown in Tables I.–IV., and in Fig. 2. Group 1, fed on foliage required an average of 16 days, whereas group 2, fed on tubers and fruit required an average of 13 days. Evidently, to accumulate sufficient nutrients necessitated a more extensive feeding on foliage than on tuber or fruit. The experiment with group 3, fed on potato foliage for six days followed by a period of feeding on sliced non-solanaceous foods, resulted in, first, an increase in the number of feeding days essential for nutrient storage to an average of 23.6 days; second, a mortality of 23 per cent.; third, unsuccessful hibernation of 32 per cent. (which died during the process).

TABLE I.

NUMBER OF DAYS FOR BEETLES FED ON FOLIAGE TO ENTER HIBERNATION.

Potato.		Tomato.		Eggplant.	
Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.
75.....	14	100.....	15	80.....	11
25.....	21			20.....	20
Average.....	17.5		15		15.5
Cages Receiving No Water.					
80.....	11	10.....	11	90.....	18
10.....	13	80.....	13	10.....	20
10.....	Died	10.....	Died		
Average.....	12		12		19

The general effect with group 4, fed on sliced non-solanaceous foods exclusively and those members of group 1, which were fed on pepper plants or its fruit, was one of starvation. Some died after 7 days; others lived for 41 days but were unable to hiber-

[1] Potato beetles hibernating in July or November exhibit the same physiological reactions.

TABLE II.

NUMBER OF DAYS FOR BEETLES FED ON TUBERS AND FRUIT TO ENTER HIBERNATION.

Potato.		Tomato.		Eggplant.	
Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.
80.....	13	10.....	11	50.....	11
20.....	15	80.....	13	50.....	18
		10.....	Died		
Average.....	14		12		14.5
Cages Receiving No Water.					
80.....	11	10.....	11	10.....	11
20.....	13	90.....	18	90.....	13
Average.....	12		14.5		12

TABLE III.

NUMBER OF DAYS FOR BEETLES FED ON FOLIAGE FOLLOWED BY SLICED TUBERS AND NON-SOLANACEOUS FOODS TO ENTER A STATE OF HIBERNATION.

Potato.		Squash.		Cucumbers.		Beets.		Carrots.	
Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.
75.....	15	80.....	25	40.....	15	40.....	13	40.....	15
25.....	25	20.....	35	50.....	21	50.....	38	10.....	38
				10.....	Died	10.....	Died	50.....	Died
Average..	20		30		18		25.5		25

TABLE IV.

BEETLES FED ON NON-SOLANACEOUS FOODS SHOWING NUMBER OF DAYS BEFORE DEATH.

Squash.		Cucumbers.		Beets.		Carrots.		No Food.	
Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.	Per Cent. Beetles.	Days.
40.....	37	40.....	15	60.....	37	40.....	15	100....	11
60.....	45	40.....	26	40.....	39	60.....	38		
		20.....	34						
Average..	41		25		38		24		

nate. In control cages where nothing was fed, the beetles died after 11 days.

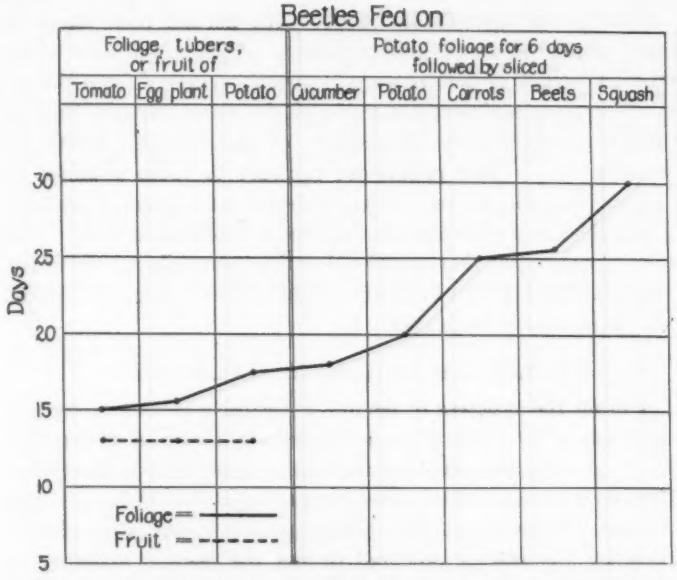

FIG. 2. Represents the number of feeding days required on different foods before the beetles entered a state of hibernation. Days are represented as ordinates, different foods as abscissa. The solid line curve shows the average number of days feeding on different foods before hibernation. The dotted line shows the average number of days feeding on tuber or fruit before hibernation.

In duplicate series of experiments conducted at the same time, certain cages received water to replace usual evaporation from the soil, others received no water. The addition of water to cages containing normal food plants decreased the feeding period from an average of 14.5 to 13.4 days. In control cages receiving only water the beetles died after 16 days. In cages containing non-solanaceous foods, the addition of water seemed to prolong the life of the beetles without inducing hibernation.

Breeding Activities.

It is perhaps essential to emphasize that potato beetles of the first generation used exclusively in these investigations indicated

no breeding activities before dormancy set in. The beetles hibernated during July and August and remained in that state until the following season. Other experiments conducted by confining first generation beetles with normal food plants in field cages, produced similar results. During fall and winter these hibernating beetles were frequently removed from the soil in the cages, and sections made of the testes revealed mature sperm in abundance. Dissections of the females yielded immature ova. These results are contrary to those obtained by Tower (24), who states, "The first brood on emergence feeds for a few days and then deposits eggs for a second generation. The second generation does not develop the germ cells nor show any reproductive activity until after it has passed through a period of hibernation or æstivation."

Metabolism During Feeding Experiments.

During the progress of feeding a veritable storing of food in the form of fat takes place in the adipose tissues of the animal (Fig. 4). It has been pointed out above that a numerical difference in days exists, for nutrient storage when different foods are fed. From metabolism determinations it seems possible to correlate the results procured during the feeding experiments with the oxygen consumption. In Fig. 3, the oxygen intake per gram organism in cubic millimeters per hour is shown. From an examination of this figure it is evident that a reduced oxygen consumption takes place when tuber or fruit material, as compared to foliage, is fed. Obviously then the process of converting tuber material required less oxygen. The analysis of food plants, given in Table V., shows the amounts of carbohydrate and fat

TABLE V.

SHOWING ANALYSIS OF FOOD PLANTS IN PERCENTAGE.

Food Plant.	Water.	Protein.	Fat.	Carbohydrate.
Beets	87.5	1.2	0.1	9.4
Carrots	88.2	0.7	0.4	8.9
Cucumbers	95.4	0.6	0.2	3.0
Eggplant	92.9	0.9	0.3	4.9
Potato	78.3	1.7	0.1	17.7
Squash	88.3	1.1	0.5	8.6
Tomato	94.3	0.7	0.4	3.8

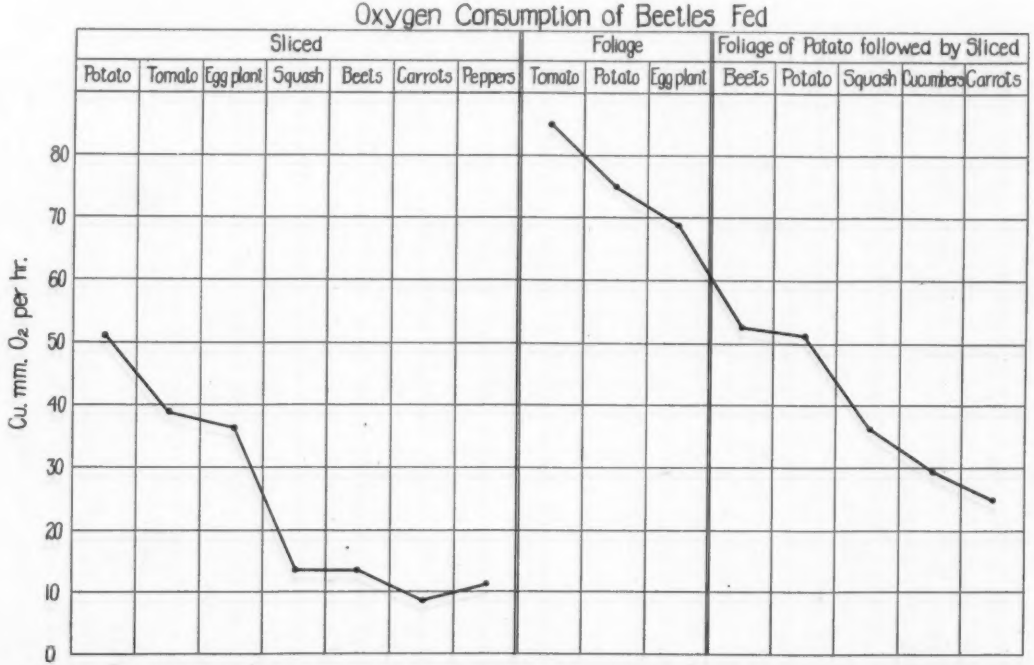

FIG. 3. Ordinates shows the oxygen intake in cubic millimeters per hour per gram body weight of beetles fed on different foods represented as abscissas. By comparison it is noted that distinctive differences in oxygen consumed are evident when potato beetles are fed tubers and fruit as compared to foliage of the same plants. This figure also shows the low oxygen intake when fed non-solanaceous foods.

present in non-solanaceous foods as compared to potato tuber and foliage. It is known that a diet rich in carbohydrates, as for example, turnips or potatoes, increases the proportion of lower fatty acids, while a diet rich in proteins diminishes this proportion. If the percentage of the lower fatty acids increases with the amount and kind of food, this fact may explain the results shown in the above experiments, since tuber material is rich in carbohydrates.

It was previously pointed out that members of group 4, fed non-solanaceous food plants and those beetles fed on peppers, in general, gradually starved. The respiratory metabolism of such animals manifests an extremely reduced oxygen consumption and (in other experiments to be discussed later) a decreased CO_2 output. In control experiments, where nothing was fed, the oxygen intake similarly was exceedingly diminished.

Preparation for Hibernation.

Of significance are the variations in weight recorded during the preparation for hibernation. First generation beetles as they emerged from pupation and before feeding averaged 0.1260 grams in weight. After feeding on foliage of the potato plant for ten days, the weight averaged 0.2627 grams. A state of quiescence or *prehibernation*[2] followed, varying from three to five days and was accompanied by a reduction of the water content and elimination of the waste material from the digestive tract, giving an average weight of 0.1879 grams. The increase in weight accounts, in part, for the accumulation of reserve nutrient material in the adipose tissues, and may, in addition, indicate growth of somatic and germinal cells. Furthermore, during *prehibernation* a gradual lowering of all vital activities takes place, the beetles afterwards entering the ground to hibernate.

Determinations of the water and fat content were made upon groups of beetles as shown in Fig. 4. From an examination of these it is evident that during active feeding, the average water and fat content was 76.4 and 7.8 per cent. respectively. During *prehibernation*, the average water and fat content was 59 and

[2] The term *prehibernation* is used to designate the quiescent state that occurs when feeding ceases and before the beetles burrow in the ground.

20 per cent. respectively. When the beetles burrowed in the ground the water content averaged 56 per cent. and the fat content 29 per cent. The results of these experiments indicate quite clearly that a loss of 20 per cent. in the water content and a gain of slightly over 20 per cent. in the fat content took place. Tower (24) obtained with this species a reduction of 27 per cent. in the water content. With hibernating grasshoppers Bodine (4) found a reduction of 13 per cent.

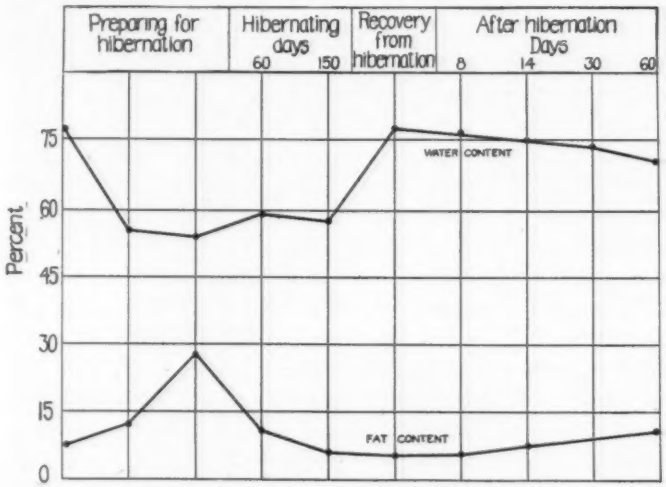

FIG. 4. Ordinates show the percentage of water and fat content of potato beetles, before, during and after hibernation. Note the reduction in the water content and increase in fat content as the beetles enter hibernation, and the extensive depletion in fat during the first few months as compared to the remaining period of hibernation.

The view commonly held is that a diminished water content makes protoplasm able to withstand extremes of temperatures. With potato beetles this does not appear to be valid, since invariably they must burrow in the ground to a considerable depth (10–18 inches) presumably to successfully escape extremes of temperatures. Experimentally the influence of greater variations in temperatures was determined by permitting only several inches of soil to intervene between the dormant beetles and the outside air. The results of such treatment upon hibernating

beetles during the winter, produced a mortality of 100 per cent. In this species a reduction of the water content probably does not proceed to a sufficient degree to prevent injury by freezing temperatures, hence necessitating their further protection by burrowing in the ground.

During the progress of hibernation, continued investigations have shown that the water content remains practically constant, although with a general diminution of fat, a slight increase in the water content may follow. Extremely significant is the excessive depletion in fat that obviously takes place during the first two months (51 per cent.) as compared to the remaining five months (49 per cent.) of hibernation.

In this connection it is of interest to cite, for comparison, the results of other investigators concerning fat consumption during hibernation. Victoroff (26) with frogs found a consumption of 23 per cent. Investigators with mammals demonstrated the prevalence of a large fat content which gradually disappears during the period of torpidity. Thus Voit (27) found that in the marmot the adipose tissue was more than 30 per cent. of the weight of the body. Valentin (25) found the adipose tissue in the marmot contributed about twenty times as much food as the hibernating gland. He also observed that the depletion in fat which occurred during the first few months was greater than after five months hibernation. Similarly Carlier (8) found that almost one half of the fat disappeared in the hedgehog during the first few months of dormancy. It is of interest to discover this general conformity between a mammal and an insect in the utilization of nutrient material during the course of hibernation.

Hibernating Gland.

Many investigators mention a hibernating gland which is said to be commonly found in mammals (hedgehog, marmot, bat, shrew, rat, mole, beaver, squirrel, weasel, martin, badger, rabbit, guinea pig, cat, dog and even in man). Rasmussen (18) who has reviewed the literature on this subject extensively, is of the opinion that it is distinct from adipose tissue. Sheldon (22) recently concluded that "the so-called hibernating gland is essentially a form of adipose tissue which retains its embryonic characteristics for a more or less indefinite length of time, . . .

it intergrades with ordinary adipose tissue and under favorable conditions is transformed into it."

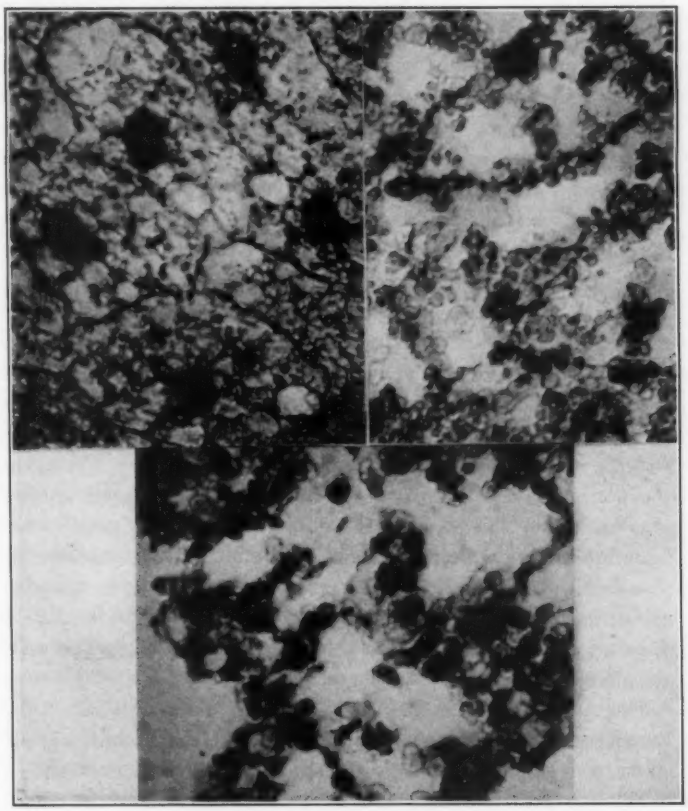

FIG. 5. Upper left. Section of the fat body or adipose tissue of potato beetle before hibernation. Note the numerous fat vacuoles partly masking the large nucleus located centrally within the cell, and the minute granules dispersed among the vacuoles. Flemming fixation, stained with hematoxylin. Photographed with achromat 2 mm., lens, 1.3 numerical aperture with 12 × compensating eye piece.

FIG. 6. Upper right. Section of the fat body after one month of hibernation, showing the decreased size of the fat vacuoles and nucleus, the large and prominent albuminoid granules grouped along the periphery of the cell exhibiting open spaces. Staining and enlargement as in Fig. 5.

FIG. 7. Section of the fat body after five months hibernation, showing the almost complete absence of fat vacuoles. The prominently large albuminoid granules are grouped in masses, the absence of cell outline of the fat body and the almost entire disintegration of the nucleus. Staining and enlargement as in Fig. 5.

Whether it is ordinary adipose tissue or a special gland that functions in the mammal as a storage for nutrient material, with the potato beetle, and perhaps with insects in general, it seems quite certain that adipose tissue serves that purpose. To determine some of the morphological changes that take place during hibernation sections were made of the adipose tissues of the potato beetle. During *prehibernation* (Fig. 5) the sections show it to be composed of regular cells, oval in general outline and filled with conspicuous fat vacuoles of more or less uniform shape and size which extensively overlap and partly mask the actual contour of the large nucleus. Barely distinguishable are diminutive albuminoid granules dispersed among the vacuoles. The sections of beetles in hibernation for one month (Fig. 6) are strikingly different in appearance from those of non-hibernating ones. In the former we find that the fat vacuoles have become much smaller and that the nucleus has undergone disintegration and diminution in size. The most striking objects are the albuminoid granules aggregated along the periphery of the cells of the fat body, or clustered around the nucleus. The sections of adipose tissues of animals in hibernation for the longest period (six months) show clearly the prominently large albuminoid granules clustered in groups, exhibiting many open spaces (Fig. 7). Indications of a dissolution of the cells of the fat bodies and a dispersal of the albuminoid granules are quite evident.

The author compared Figs. 5–7, with those published by Rasmussen and Sheldon of sections taken from the hibernating gland in mammals, and the similarity is indeed very striking.

The existence of albuminoid granules or uric acid concretions indicates an active metabolism in the fat body. According to Lang (15) the fat body of larvae of insects is rich in fat and poor in concretions of uric acid before metamorphosis, while in the adult the opposite is true. Fabre (11) thinks that the adipose tissue serves the purpose of a urinary organ, since urates are formed within the cells. Both Graber (13) and Landois (14) regard it as a single many lobed lung (owing to the many fine branched tracheal endings in the fat body). Similarly Roubaud's view of the necessity of a physiological purification during

hibernation, based upon observing adipose tissue loaded with urate crystals before hibernation, must be regarded as signifying that it serves the purpose of an excretory organ.

There appears no valid reason for assigning to the fat body a urinary function. In the hibernating animal the metabolic activity of most cells is reduced to a minimum, and in the fat cells, judging from the respiratory quotient, it is most active. Hence the cells become charged with an accumulation of albuminoid granules and other products. The urates found in these cells are not an indication of their special urinary function, but of a more active metabolism at a period which serves the animal best. This may occur before or during metamorphosis, or throughout the progress of hibernation.

The Metabolism of Active, Starving and Hibernating Beetles.

A knowledge of the respiratory metabolism of active, starving and hibernating beetles seemed desirable for comparing the chemical changes involved. Of singular interest are the analogous rates of CO_2 output procured from beetles emerging from hibernation and pupation (Figs. 8–9), indicating, perhaps, a condition of physiological youngness or purification (Roubaud) as a result of hibernation. Bodine (4) found hibernating grasshoppers produced a higher rate of CO_2 output than growing animals kept at the same temperature, suggesting he states, "that animals remain young throughout the period of hibernation." The CO_2 output of potato beetles throughout the progress of hibernation invariably indicates a reduced metabolic activity, in some respects comparable with that of starving animals (Fig. 9). Indeed, there appears to be a parallelism in respiratory metabolism between starving and hibernating forms. In the former, however, the velocity of reaction of the life processes continues to function most actively and reserve substances are rapidly depleted, a condition eventually leading to the death of the animal. In the latter most of the life activities are considerably depressed and nutrient material is, therefore, used sparingly.

With older beetles, the metabolic activities are also extremely reduced, but not to the extent met with in hibernating or starving

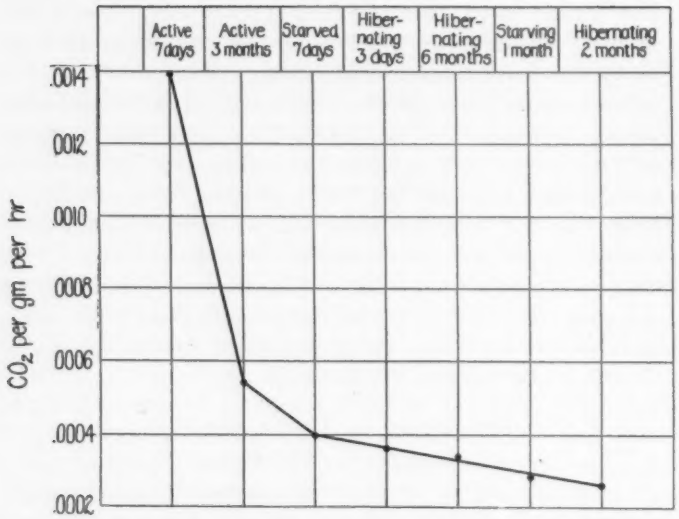

FIG. 8. Ordinates represent the CO_2 output per hour per gram body weight of organisms. Note the reduced CO_2 output of hibernating and starving as compared to active animals, indicating a parallelism in metabolic activity between starving and hibernating beetles.

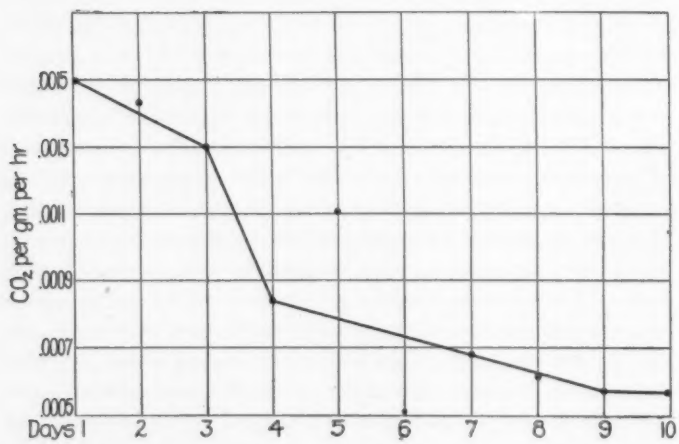

FIG. 9. Ordinates show the CO_2 output per hour per gram body weight of beetles during the progress of starvation until death. Abscissas represent days.

forms. The overfed and old state of the cell, as suggested by Baumberger (1) has perhaps reduced permeability to a wide degree. In old age or pathological conditions, according to Mathews (16), an accumulation of inactive ingredients or of acid in the cells, retards metabolic activity. In a measure, we are thus enabled to interpret the specific differences in metabolism that may take place under varying conditions in the organism. Especially noteworthy is the utilization of reserve nutrient material in the hibernating animal discussed further on.

Respiratory Quotient.

It is well known, experimentally, that when substances catabolized are chiefly fat, a reduced respiratory quotient results, whereas the catabolizing of carbohydrates and protein produces a higher quotient. According to the investigations of Dubois (10), Pembrey (17), Weinland and Riehl (29), Valentin (25) and others, reduced respiratory gas exchanges in the marmot occur during hibernation with strikingly low respiratory quotients (0.44–0.72). The views advanced to explain a quotient not consistent with the utilization of fat are many. Certain investigators (Dubois, Pembrey, Valentin) perceived that although mammals consumed no food during dormancy an increase in weight very often took place. It was, therefore, assumed that the oxygen consumed, greater than is necessary for fat oxidation, was in some way retained in the animal. Dubois also discusses building of acetone which he found accumulated in the blood-urine of hibernating mammals. It is known that of the normal fatty acids from butyric to decoic acid, only those with an even number of carbon atoms give rise to a marked increase in acetone formation. Dakin (9) and others have shown that acetone is derived from the decomposition of acetoacetic acid. This supports Knoop's theory of the B-oxidation in which two or some multiple of two carbon atoms are lost during oxidation, and offers a possible explanation that intermediate stages supervene throughout the process of the utilization of fat. Furthermore, Weinland and Riehl claim that during awakening from dormancy the marmot needs carbohydrates, and that the required carbohydrates come partly from substances produced

in the body during hibernation. But it is not certain whether they are produced from fat, protein, or both. Voit (27) holds that fat may form sugar, which in turn can be stored up as glycogen.

The respiratory quotients obtained with the potato beetle demonstrate a wide degree of variation during different periods of hibernation. The respiratory quotient is lowest during the

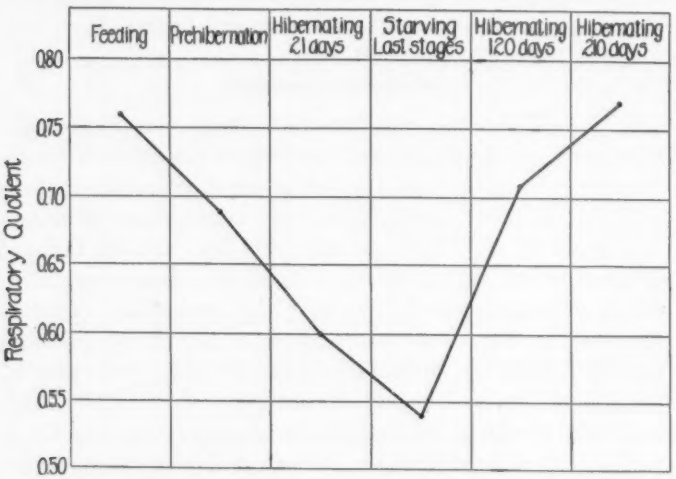

FIG. 10. Ordinates show the respiratory quotient based on respiratory metabolism (CO_2 output and oxygen intake) of active beetles and of those after varying periods of hibernation as compared to starving animals.

first few months of dormancy and increases progressively. A noteworthy increase in the quotients results when the animals awaken to activity (Fig. 10). This may agree with the theory brought forward by Weinland and Riehl, that carbohydrate is being oxidized for the purpose of awakening. On the other hand, during acute stages of starvation, potato beetles evince an exceedingly reduced respiratory quotient (0.54). Dubois has shown that a hibernating mammal loses as much in weight in 160 days as a starving animal in 12 days. Since it is recognized that reserve nutrient materials are rapidly depleted during starvation it is possible, perhaps, to correlate the diminished respiratory quotient noted during the early phases of hibernation

with the immense decrease in the fat content which likewise results at this period.

Recovery from Hibernation.

In the preparation for hibernation the potato beetles pass through a quiescent phase which has been designated as *pre-hibernation*. Likewise on emergence from dormancy there is a period of quiescence known as recovery. From results obtained on respiratory metabolism during recovery and before feeding, it is evident that the increase in CO_2 output is very gradual, lasting for six days (Fig. 11). The examples upon which the

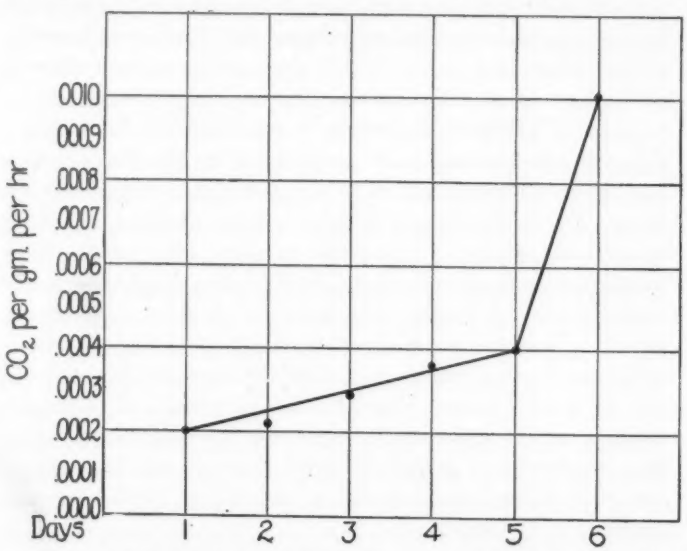

Fig. 11. Ordinates show the average CO_2 output per hour per gram body weight of beetles during recovery from hibernation. This figure is based on group of beetles hibernating for six months.

above figure is based, comprised a group of beetles in hibernation for six months. After varying periods of dormancy, if potato beetles are removed from the ground and placed with food, we find they require from six to thirty days for recovery before feeding. Those that have been in hibernation for over two months require 30 days, those for three months 12 days, and

those five or more months 6 days. During the interval of restoration they are decidedly inactive, negatively heliotropic and do not feed.

Extensive experiments carried out during the winter with the view of hastening recovery proved fruitless. In every instance hibernating beetles when removed from the ground and placed with food persistently indicated tendencies to burrow themselves in again. In those cages not provided with soil the beetles remained inactive on the side or bottom of the cage without feeding.

Both Bodine (4) and Sanderson (20) found that the rate of recovery was quickest with animals brought in later in the hibernating period. Sanderson, however, concluded that subjecting hibernating forms to low temperature had the effect of producing a more complete rest than at a higher temperature. In view of additional experiments performed by the author a different interpretation may be attributed to the above results. For example, beetles reared in the greenhouse hibernated the latter part of March and were kept at a comparatively high temperature (65–95° F.) in the greenhouse. On July 11, they were found issuing from the ground in the cages, exhibiting normal activities such as feeding, mating, etc. In these experiments, therefore, cold was not essential for a complete rest. Furthermore, the depth at which potato beetles normally hibernate in the soil (10–18 inches) precludes the assumption of a specific influence of low temperature, or of the necessity of subjecting them to prolonged periods of cold. On the contrary, experimental evidence demonstrates a rhythmical period of rest irrespective of temperature.

Adjustment of the Water Content.

Of paramount importance to the animal during recovery and before feeding is possible, is an adjustment of its water content to normal. The need for actual contact of the animal with water at different intervals seems to be essential. Whether moisture may also be absorbed through the integument is not definitely known.

In the following experiments beetles recovering from hibernation were kept in cages with abundance of food under the

same temperature conditions in the greenhouse. One lot frequently received water, the other lot was not watered. The addition of water (a drenching of the cage) had an activating effect, since the beetles eventually became active and fed normally on the plants. In those cages not receiving water the beetles were apparently unable to recover and finally perished. The lack of actual contact of the animal with water evidently hindered recovery.

Dissected hibernating beetles invariably revealed accumulated waste products in the rectum. In normal animals its expulsion is necessary before activity and feeding is possible. It seems reasonable that water actually is imbibed by the animal during recovery to aid in the elimination of inert substances from the digestive tract.

Catalase and Oxidase Activity.

It seemed desirable to determine if other factors concomitant with a lowered metabolic activity are involved in the hibernating animal. There are many facts in the literature tending to show that the power of decomposing hydrogen peroxide and the power to blue guaiacum by the aid of peroxide are the specific property of certain substances. Since the recognition of catalase as a specific enzyme, its occurrence and distribution in various animal and vegetable tissues has been investigated by a number of observers.

Certain investigators are of the opinion that the accumulation of hydrogen peroxide would undoubtedly prove harmful to the organism, and that the function of the catalase is to destroy the hydrogen peroxide as fast as it is formed. Others hold that the function of catalase may be to prevent the excessive oxidation of organic substances in the living cell. Schoenbein (21), however, has shown that substances which can bring about the decomposition of hydrogen peroxide catalytically, can also greatly increase its oxidizing power, and in proportion as a substance is able to decompose the peroxide so also it can accelerate oxidation. The power to decompose hydrogen peroxide is held by Spitzer (23) to be a measure of the oxidizing power of various animal tissues. Dakin (9), however, is of the opinion

that there is no trace of evidence to prove that catalase is directly concerned with oxidation, since catalase, he states, "only liberates inactive molecular oxygen when decomposing hydrogen peroxide." Burge (7) found a quantitative relation between the amount of catalase and oxidation; an increase of oxidation in young and a decrease in old animals. Similarly Bodine (5) with certain insects obtained a decreased catalase content with increasing age and with animals subjected to starvation.

The author made determinations of the activity and total content of catalase in hibernating, starving, young and old potato beetles, using for this purpose the same methods described by Burge. The results of these experiments are graphically represented in Figs. 12–13. With hibernating animals the total catalase content per gram body weight is lower than with either

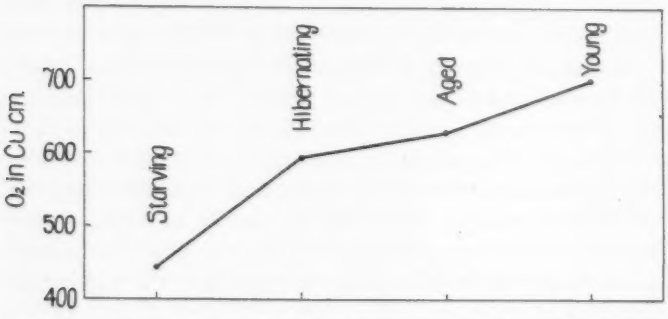

FIG. 12. The average total catalase content per gram body weight of different groups of beetles as measured by the oxygen evolved. Ordinates show the oxygen evolved in cubic centimeters.

young or old beetles; starving animals indicate the lowest catalase content. In Fig. 13, the catalase activity per gram body weight per minute is recorded and strikingly indicates a seeming parallelism in catalase activity between hibernating and starving animals. Moreover, the extremely reduced catalase activity indicates a correlation with a diminished respiratory metabolism in hibernating and starving animals.

Experiments to determine the oxidase activity were made by using guaiac, P-diamino benzene with peroxide upon tissues, organs and body fluids. Upon hibernating animals the reaction

were negative since no typical characteristic blueing of guaiacum took place. Similar tests performed with active and starving beetles, gave striking reactions (blueing of guaiacum) with esophagus, stomach, tip of rectum, tissues, body fluids, testes and immature ova.

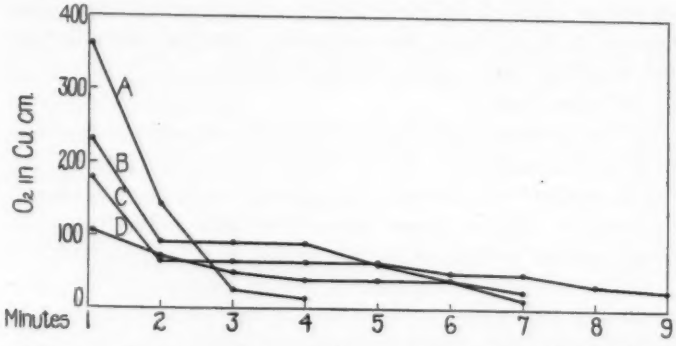

FIG. 13. The catalase activity per minute per gram body weight of organism. Ordinates show the oxygen evolved in cubic centimeters, abscissas as minutes. *A*, young beetles; *B*, aged beetles; *C*, hibernating beetles; *D*, starving beetles.

Although the catalases and oxidases may possess specific functions in the active animal, in the hibernating forms these enzymic activities obviously become greatly reduced or seemingly absent.

SUMMARY.

1. The results of the food experiments indicate that potato beetles of the first generation when fed on tubers and fruit of the potato, tomato, and eggplant require 13 days feeding to enter hibernation, while those fed on foliage of the same plants need 16 days. Beetles fed on potato foliage for six days followed by a non-solanaceous food required 23.6 days feeding before hibernation, and produced a mortality of 55 per cent. Those fed on pepper plants or on non-solanaceous foods do not hibernate, but die of starvation.

2. Respiratory metabolism determinations show that the oxygen consumption of beetles fed on tubers or fruit of solanaceous plants is less than of those fed on foliage. When fed on non-solanaceous foods, the reduced oxygen intake recorded was

comparable with the oxygen consumption of starving animals. A reduced CO_2 output occurs throughout the progress of hibernation.

3. Preparation for hibernation follows a period of extensive feeding and consists in an accumulation in the adipose tissues of 29 per cent. fat. During prehibernation a reduction of the water content of 20 per cent., the elimination of waste products from the digestive tract and a lowered metabolic activity of the animal take place.

4. The greatest depletion in fat occurs during the first few months of hibernation. In the metabolism of the fat body, the fat is replaced by albuminoid granules and other products. Sections of adipose tissue before and after hibernation are comparable to sections of the hibernating gland of mammals.

5. The respiratory quotient is lowest during the first few months of hibernation and highest at the termination of dormancy (0.60–0.76). In hibernating animals possessing insufficient nutriment and in starving animals, the respiratory quotient is exceedingly low (0.54). There appears to be a parallelism between a rapid depletion of reserve food and a low quotient.

6. Recovery depends upon the length of time potato beetles spend in hibernation. It is more rapid with those in hibernation for the longest time. Actual contact of the animal with water seems necessary to restore its water content before feeding is possible.

7. Catalase activity of hibernating beetles is greatly reduced and their total catalase content is lower than that for old or young active beetles. There appears to be a correlation between the reduced catalase activity and diminished respiratory metabolism in hibernating and starving animals.

Although active beetles give very striking oxidase reactions, no characteristic blueing of guaiacum was obtained with any organ, tissue, or body fluid of hibernating forms.

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